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14. ABSTRACT

We hypothesize that the ability to grow an axon over CSPGs is intrinsic to adult zebrafish brainstem neurons and entails the expression of a distinct set of genes. This premise will be addressed using in vitro adult zebrafish brainstem cell culture systems and in vivo adult zebrafish spinal cord injury model systems. In cultures we have observed three distinct populations of brainstem neurons with regard to their response to chondroitin sulfate proteoglycans (CSPG). Some cells attach, extend processes, and remain exclusively associated with CSPG. Other cells attach outside and extend processes into CSPG-rich areas. A third kind of cell was found to attach outside and extend processes up to but never into CSPG-rich areas. In fact, these processes were clearly repelled by CSPG. We are currently quantifying different aspects of these three adult zebrafish brainstem neuron populations. Thus, these cultures mimic the in vivo behaviors of brainstem populations after SCI. In parallel to these in vitro studies, we have developed minimally invasive spinal cord transection and tracer injection techniques. These are currently employed to investigate the evolution of the scar and the time course of axon regeneration after spinal cord injury. The data from these first in vivo experiments will serve as a basis to optimize our harvest of retrogradely labeled adult brainstem neurons that did or did not regenerate their axon beyond a transection site.

15. SUBJECT TERMS

None provided.

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Contract # W81XWH-09-1-0403

Title: Molecular Determinants Fundamental to Axon Regeneration after SCI

PI: Dr. Jeffrey Alan Plunkett

Final Report of Scientific Progress from June 9, 2009 – December 9, 2010 (Months 1-18)

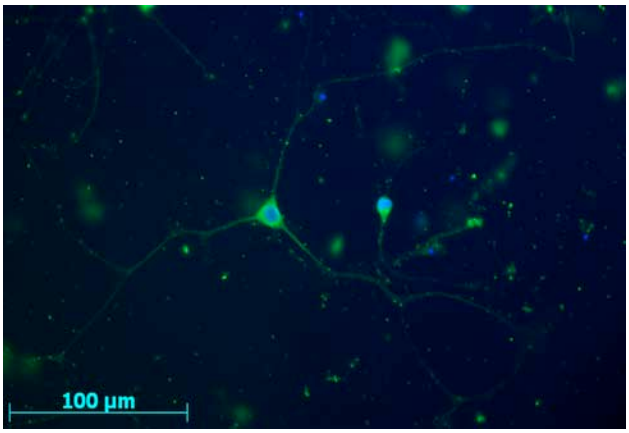
Introduction:
The zebrafish spinal cord model system is unique because of the co-existence of brainstem neurons that do (regenerators) and others that don't (non-regenerators) grow their axon beyond a spinal cord injury. These responses occur in the presence of CS-PGs, which are well-known inhibitors of axon growth in the injured mammalian spinal cord. In this proposal (the first phase of a long-term plan), we will use an *in vitro* and an *in vivo* model system to address the overall hypothesis that *the axon growth response in the injured zebrafish spinal cord is intrinsic to brainstem neurons and entails the expression of a distinct set of genes.* In Specific Aim 1, we will determine *in vitro* the effect of growth-inhibitory CSs on axon growth from primary brainstem neurons from the adult zebrafish. The experiments in Specific Aim 2 are designed to reveal the involvement of L1.1 in axon growth. In Specific Aim 3, we will identify genes that are fundamental to successful axon regeneration past a CS-PG-rich area in the injured spinal cord.

Body:

SOW: Plunkett Laboratory
Specific Aim 1: To determine *in vitro* the effect of growth-inhibitory chondroitin sulfates on axon growth from adult zebrafish primary brainstem neurons.
Experiments will be performed in established culture model systems to determine the extent of axon growth by isolated zebrafish brainstem neurons grown on substrates of purified CS. Zebrafish brainstem neurons have been cultured in the Plunkett laboratory in preliminary experiments. The dishes will be covered entirely with CS. Control cultures will have no CS (Months 1-9).

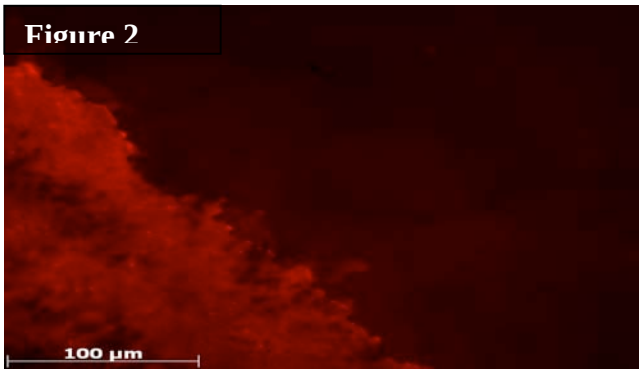
Milestones:
Month 1-3: Postdoctoral Fellow/Research Technician (tbd); Dr. Plunkett's laboratory.
Month 1-3: Establish neuronal cultures, including different coatings.

The ACURO approval for animal use was reviewed and approval was granted for work on specific aims 1 and 2 on August 24, 2009. A Postdoctoral Fellow was hired at St. Thomas University to carry out the work set forth by Aims 1 and 2. The Postdoctoral researcher is Dr. Alexis Tapanes-Castillo who had her training in the laboratory of Prof. Vance Lemmon at the Miami Project to Cure Paralysis, University of Miami, Miami, FL.



Culture conditions for the establishment of adult brainstem cultures have been accomplished in the 3 month review period. Specific concentrations for plate coating with nitrocellulose, Poly-D lysine and laminin have been established. In addition, plating densities of brainstem cells per well have been established. We are also currently working on serum-free and serum weaning procedures for the brainstem cells that will allow for an environment free of growth-permissive cues, which could interfere with our analyses. With culture techniques previously established we have also found a technique using fluorescent antibodies to label axons and brainstem cells. Using antibodies directed against tubulin we have found that under appropriate culture conditions we now can visualize cells with > 100um processes after 7 days in culture and have determined this time-frame to be appropriate to carry out the experiments. Furthermore, the fluorescent tubulin labeling has allowed us to visualize many more cells than detected previously using DIC light microscopy. A micrograph of cells labeled with antibodies against tubulin is shown in ***Figure 1****.*

In other experiments we have also established proper CS coating techniques on substrates described above. As shown in ***Figure 2****, we are able to mix CS with rhodamine dextran and so exactly detect the location of CSPGs on the culture dish substrate (note: in figure 2, CS is fluorescent red). We will be using this procedure to coat the entire well to establish the ability of brainstem cells to grow on this CS-containing substrate.*



Month 4-6: Test CS inhibitory action and loss after treatment with chondroitinase ABC.
Month 7-9: Perform Exp. #1 and #2 (all conditions simultaneously).

During this review period we have established that the use of CS (Chondroitin sulfate alone) or a CSPG (chondroitin sulfate with protein core) result in similar cellular responses in our cultures. We will be performing all experiments from this point forward using CSPGs. We have also established serum-free and serum weaning procedures for the brainstem cells that will allow for an environment free of growth-permissive cues. In cultures described above we

have observed three distinct populations of cells with regard to their response to CSPGs presented to them in culture. We have identified populations of cells that attach, extend processes and remain exclusively associated within the CSPG area on the plate. We have also observed a population of cells that attach outside and extend processes into the CSPG rich areas. Finally, we have observed a population of cells that attach outside, extend processes towards but never into CSPG areas. These processes are repelled by the CSPGs. The brainstem neurons that are inhibited by the CSPG border serves as its own control for CSPG border inhibitory efficacy. We are currently performing experiments in which we have chondroitinase ABC treated the substrates. However, because we currently have populations of cells that grow inside, and cross the CSPG barrier we feel that chondroitinase ABC treatment experiments will yield little informative data. Because we also have cells that cross into and are repelled by CSPG barriers in the same culture dish, the optic crush control experiments originally proposed will not be necessary for future experiments.

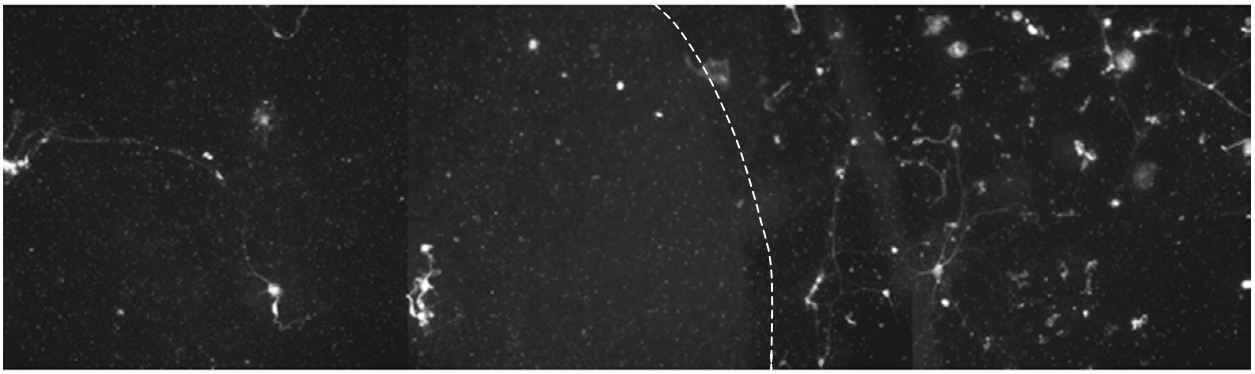


Figure 3. Montage image of cultured brainstem cellular responses to CSPG. Figure shows two of the cellular responses to CSPG. Dashed line represents the CSPG border. The left side of the dashed line is coated with CSPG and the right side of the line is absent of CSPG. The left side of figure shows a cell extending processes in an area of CSPG. The right side of figure shows cells extending processes in an area devoid of CSPG, and avoiding the CSPG border (dashed line).

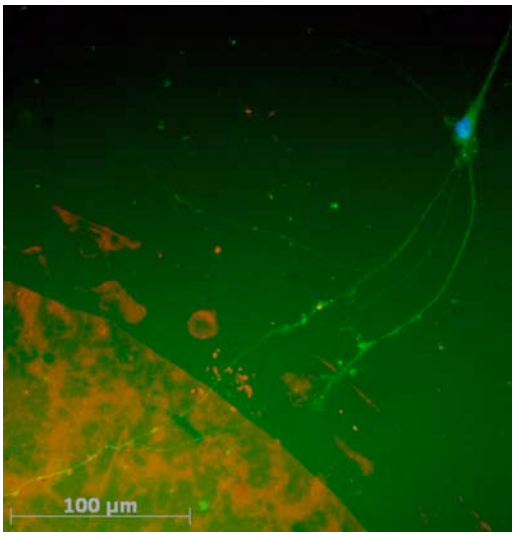


Figure 4. Triple-label immunofluorescent images of cultured brainstem cells extending processes and entering areas of CSPG. CSPG are labeled with rhodamine dextran (red) and axonal processes are labeled with anti-tubulin antibodies with a fluorescein (green) secondary. Cell nuclei are labeled with DAPI (blue). Figure clearly shows a population of adult brainstem neurons that cross into areas of CSPG. Note of added proof: within the same culture well, we observed cells that extended processes and avoided the CSPG border. This indicates that our CSPG border was truly inhibitory to other populations of cells with in the same culture well.

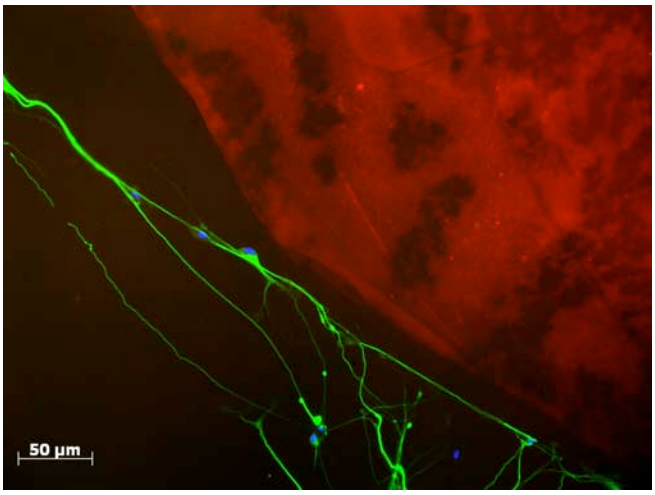


Figure 5. Double-label immunofluorescent image of cultured brainstem cells extending processes and avoiding areas of CSPGs. CSPGs are labeled with rhodamine dextran (red) and axonal processes are labeled with anti-tubulin antibodies with a fluorescein (green) secondary. Figure clearly shows a population of adult brainstem neurons that avoid areas of CSPG.

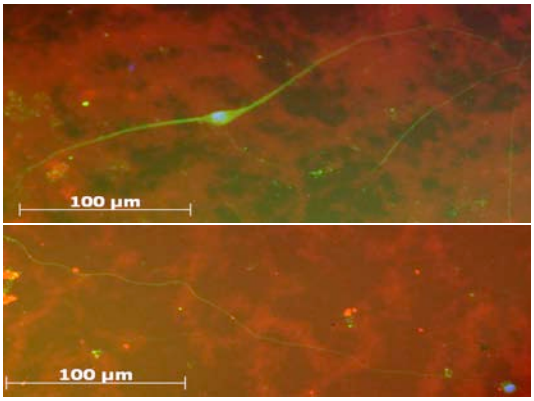


Figure 6. Triple-label immunofluorescent images of cultured brainstem cells extending processes in areas of CSPG. CSPG are labeled with rhodamine dextran (red) and axonal processes are labeled with anti-tubulin antibodies with a fluorescein (green) secondary. Cell nuclei are labeled with DAPI (blue). Figure clearly shows a population of adult brainstem neurons capable of extending processes within CSPG. Note of added proof: within the same culture well, we observed cells that extended processes and avoided the CSPG border. This indicates that our CSPG border was truly inhibitory to other populations of cells with in the same culture well.

Month 10-12: Analyze results Exp. #1 and #2. Perform Exp. #3.

In cultures described above we have observed three distinct populations of cells with regard to their response to CSPG presented to them in culture. We are currently performing experiments in which we have quantitatively analyzed neuronal populations and their interactions with CSPG borders. Plates received spots of CSPG followed by laminin coating. We have identified populations of cells that attach and extend processes that remain exclusively associated with CSPG ***(Figures 6 and 7)****. These cells with neurites appeared to prefer substrates of CSPG. We have also observed a population of cells that attach outside and extend processes into CSPG-rich areas* ***(Figures 4 and 7).*** *Finally, we have observed a population of cells that attach outside the CSPG area and extend processes up to the CSPG border but appear to be repelled by the CSPG* ***(Figures 5 and 7)****.*

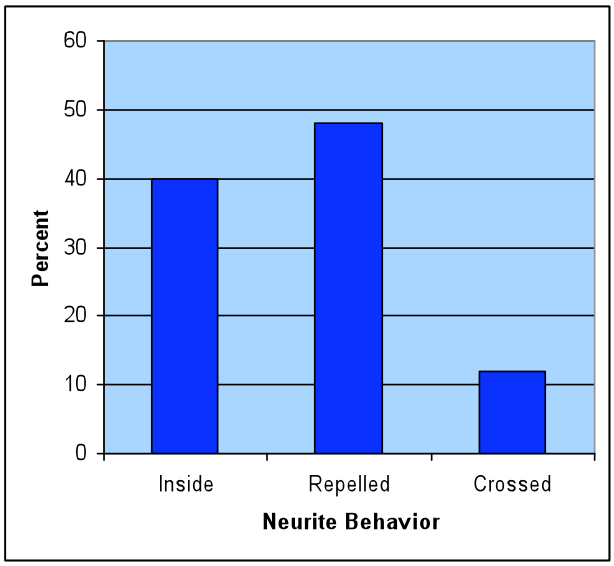


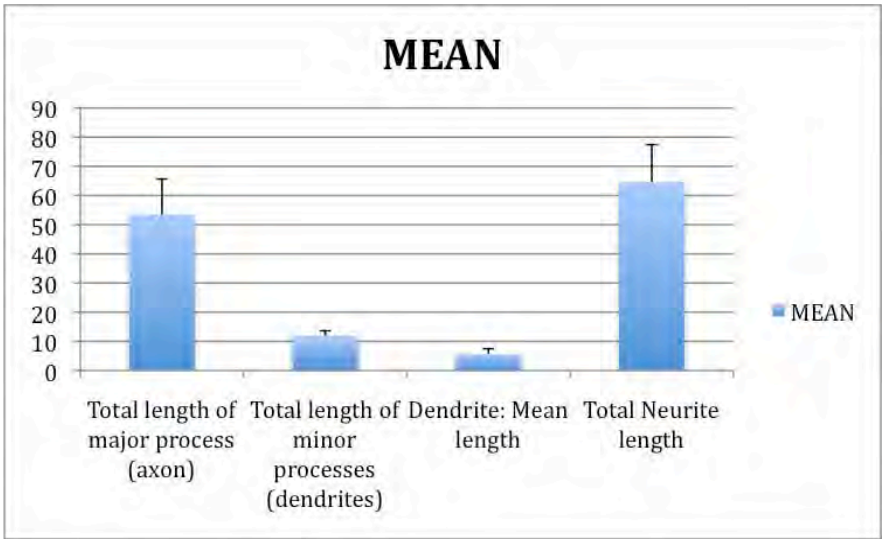
Figure 7. Cultured brainstem cellular responses to CSPG. Quantitative data were obtained from cultures containing 1ug of CSPGs. In total 100 cells were included in the analysis. All analyzed cells were within 20 μM of a CSPG border. Graph demonstrates that of the total number of neurites analyzed, 12% of the neurites crossed from areas of laminin alone into CSPG containing areas (*Crossed)*. Within the same cultures, 48% of the neurites were repelled at CSPG borders *(Repelled)*. These results indicated that CSPGs are inhibitory to a sub-population of brainstem neurons. A population, 40% of brainstem neurons was also observed that exclusively associated with CSPG *(Inside)* and remained in the CSPG area. Note of added proof: when 2ug spots of CSPG were used 99% of cells were repelled, with 1% inside and 0% crossing.

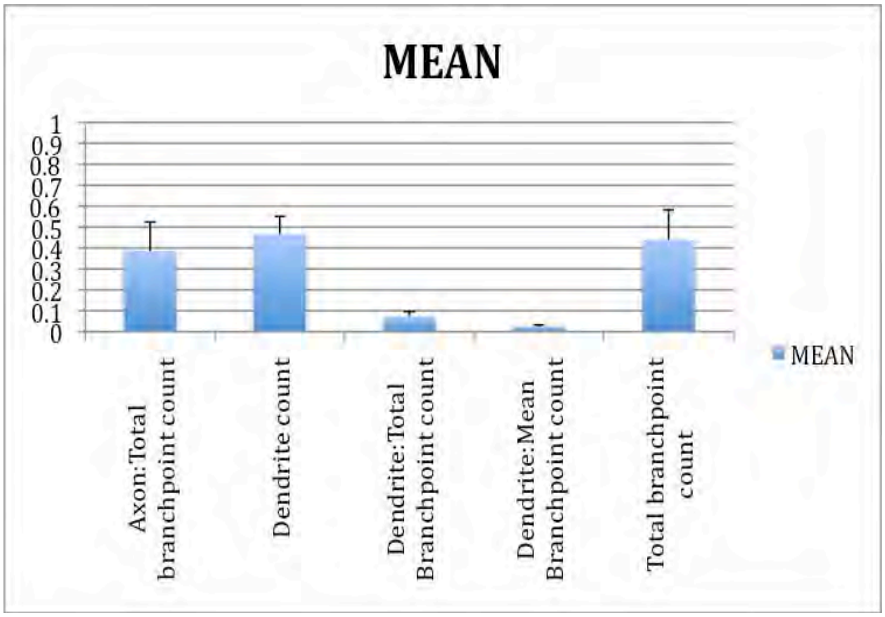
Month 13-15: Analyze results Exp. #3. Start manuscript. Prepare follow-up experiment.

During this review period we have continued performing experiments as were planned in our original proposal. The outcomes thus far support our hypotheses. We are well on our way in preparing a manuscript for submission to a peer-reviewed journal. In addition, we have determined that characterization of brainstem culture conditions is necessary for the publication of methodologies associated with our cultures. To this end we have dedicated time in this quarter to the characterization of adult brainstem cultures. We have quantitatively analyzed cultures at various time-points to assess neurite outgrowth properties. This work establishes a base-line of culture cell behavior following plating under CSPG-free conditions. We have concentrated on analysis of neurite outgrowth with the establishment of branching. Below is a summary of this data at various time-points in complete media.

Neuronal morphology of 1 day cultures in complete serum-containing media

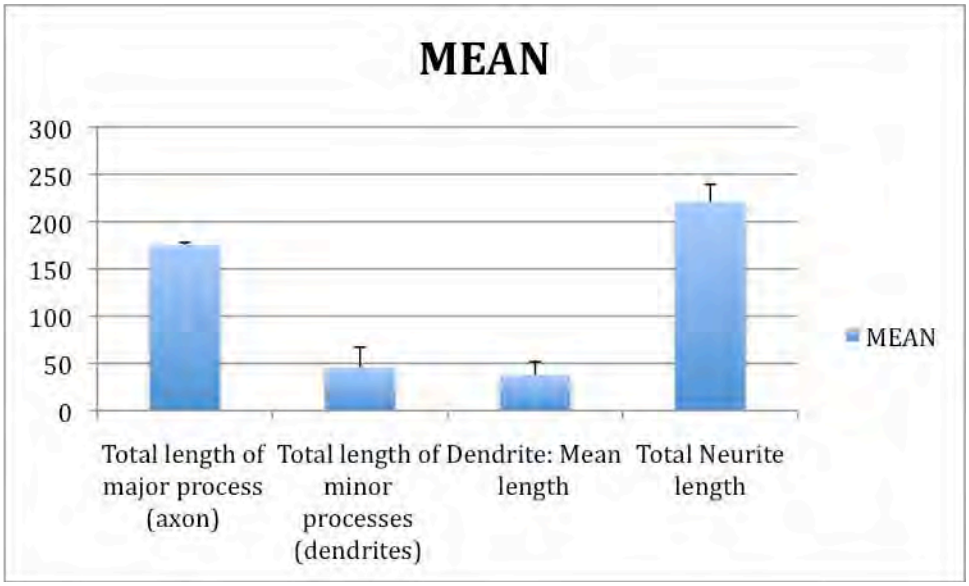
Data below are from 3 independent experiments.
Each experiment had at least 30 cells.
Data from a total of 145 cells was used to compile data.

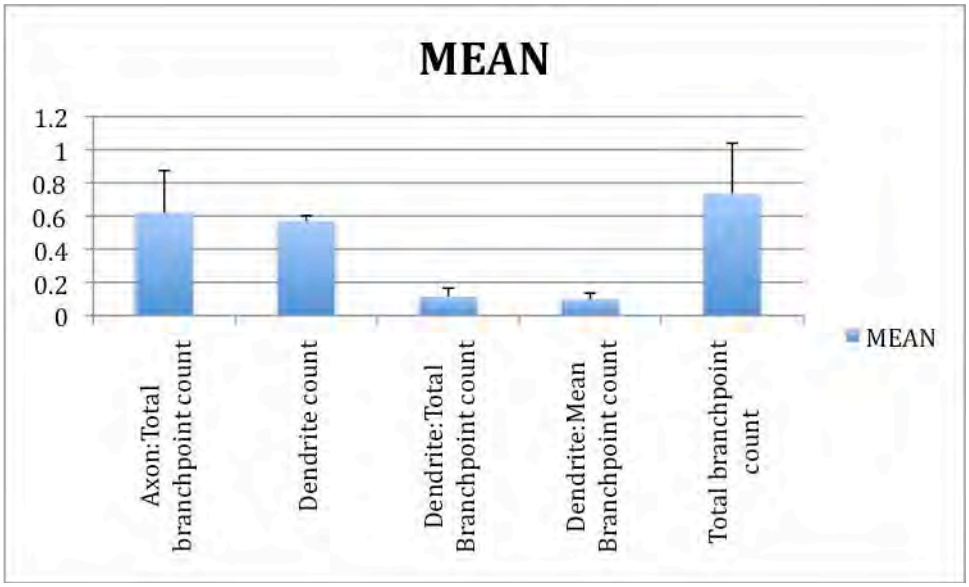




Neuronal morphology of 3 day cultures in complete serum-containing media

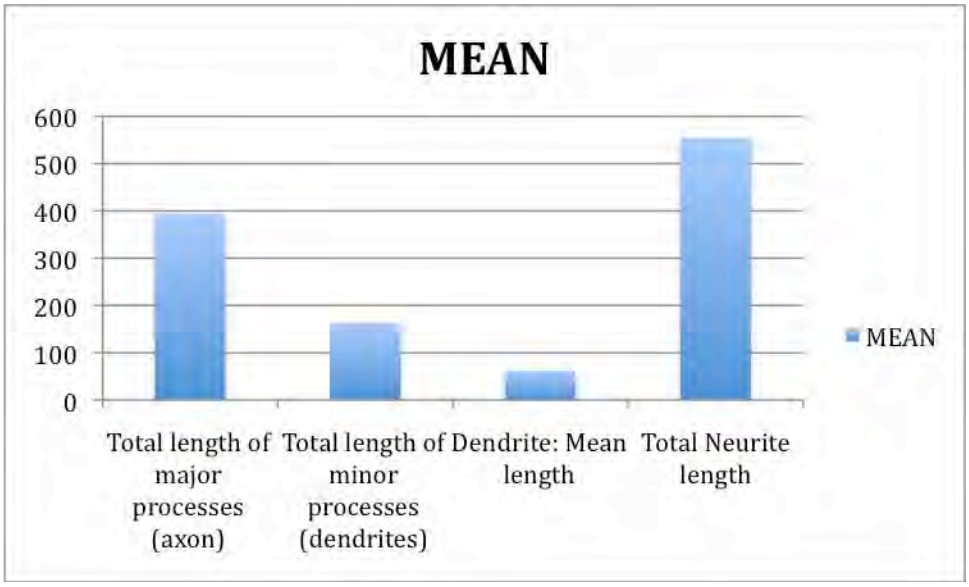
Data below are from 2 independent experiments.
Each experiment had at least 20 cells.
Data from a total of 47 cells was used to compile data.

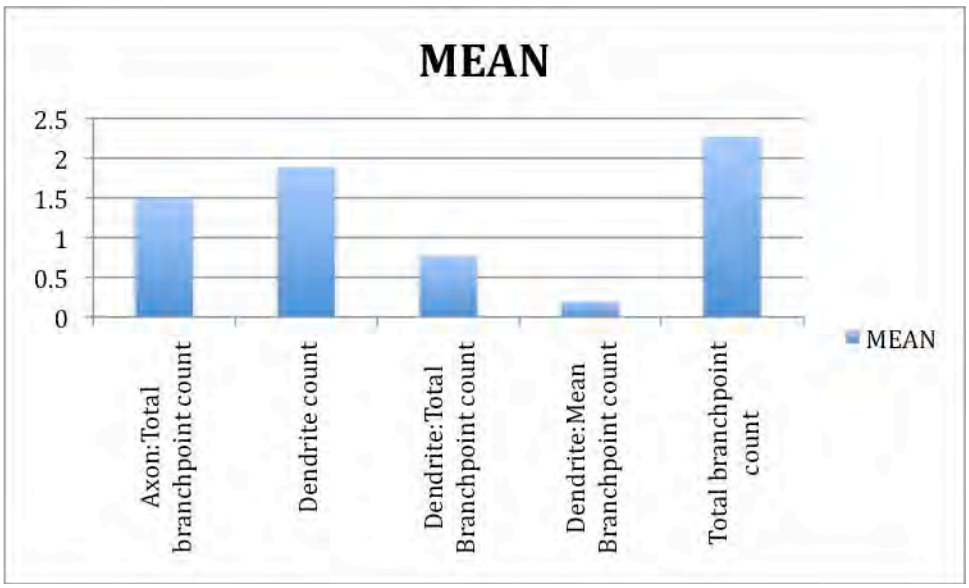




Neuronal morphology of 7 day cultures in complete serum-containing media

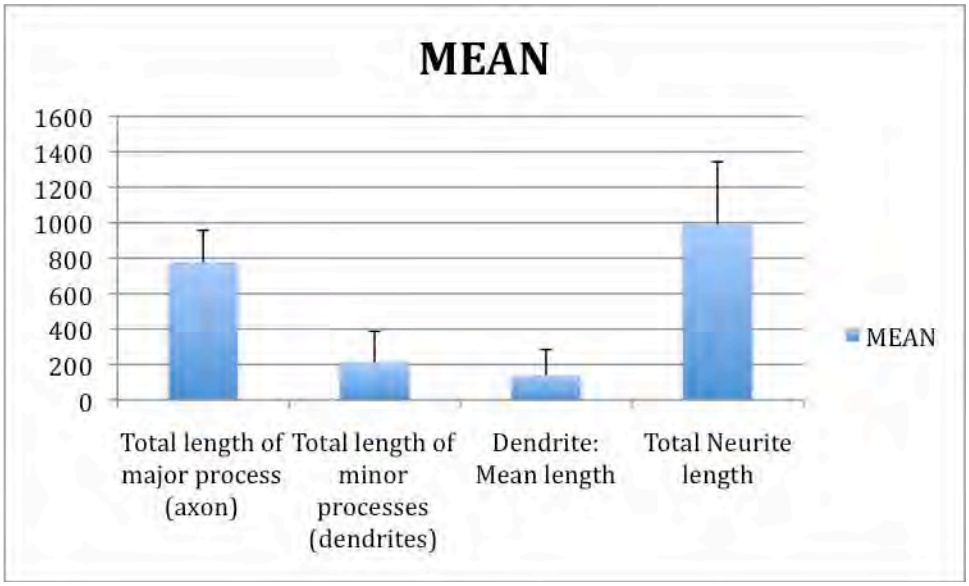
Data below are from 1 experiment.
A total of 26 cells were used to compile data.
Note: Analysis is continuing and statistical analysis will be added as more experiments are analyzed.

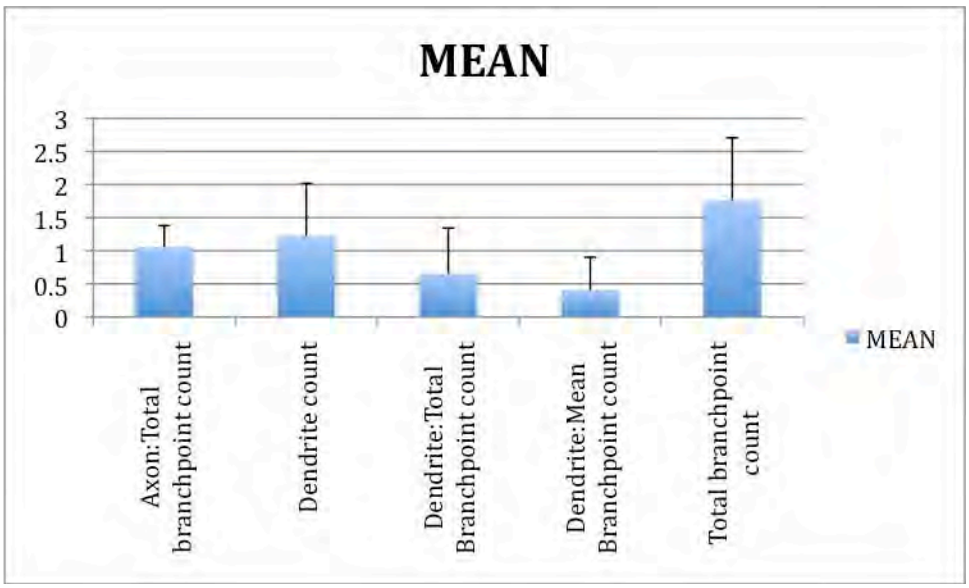




Neuronal morphology of 14 day cultures in complete serum-containing media

Data below are from 2 independent experiments.
Each experiment had at least 12 cells.
Data from a total of 26 cells was used to compile data.





*Analysis of data presented above represents cells grown in **complete serum-containing** media in the absence of CSPG influences. In summary, we can determine that brainstem cells have an average process length of 60μM one day following plating with the average increasing to 1000μM by day 14.*

Month 16-18: Finalize/submit manuscript. Start follow-up experiment.

*We are currently using the above described methodology to determine a base-line analysis of cell grown in **serum-starved media**. The serum-free conditions will be directly applicable to the CSPG project as analysis of CSPG containing cultures will be performed under serum-starved conditions. Below is data presented above combined with serum-starved culture data developed in the final quarter.*

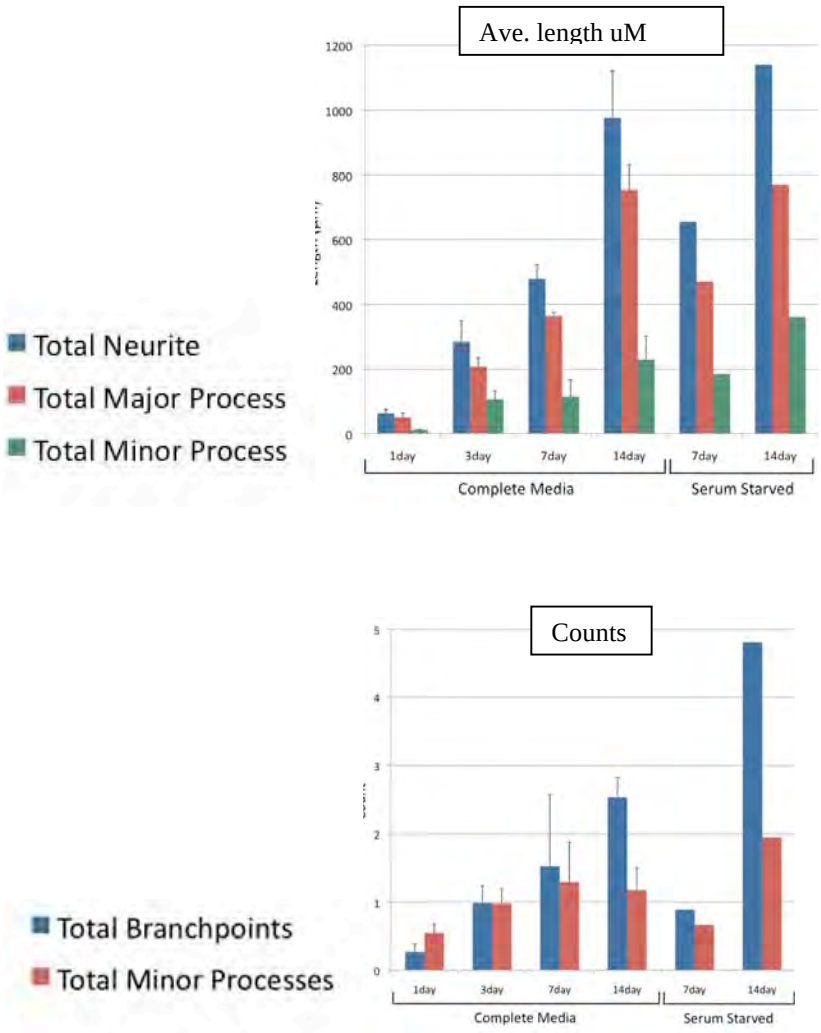


Figure 8. Combined neurite morphological analysis of cells grown in complete and serum-starved media.
Neurons were plated on a nitrocellulose, PDL and laminin substrate in complete media. After 48 hours, media was replaced with either fresh complete media or serum-free media in serum starved conditions. Error bars were obtained using the average range of two experiments or the standard error of the mean (SEM) of three experiments. All conditions had at least 20 cells per experiment except for 14day complete media and 7 day serum starved cultures.

In conclusion, analysis of culture conditions will allow us to publish base-line data about our novel adult brainstem culture system. Moreover, his type of analysis is the ground work for our CSPG containing culture analysis which is ongoing in the laboratory. We expect to complete the analysis of serum-free cultures grown in the presence of CSPGs by spring 2011. A separate manuscript is planned for our CSPG studies. Initial analysis of CSPG containing cultures reveals that cells residing exclusively in areas of CSPGs after 7 days in culture have reduced neurite length and more process branch points when compared to cells of the same age grown on laminin alone. This analysis is only preliminary and ongoing experiments will determine if this trend continues and is statistically significant.

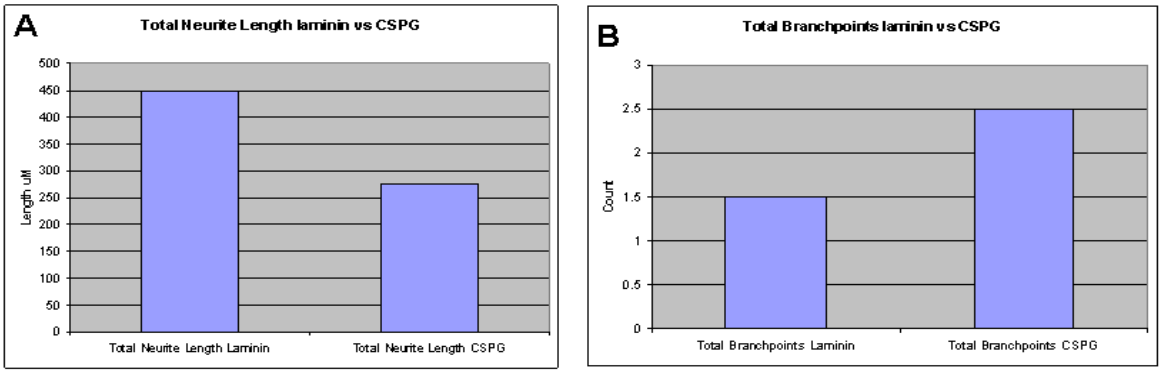


Figure 9. Initial Analysis of Neurite length (A) and branchpoints (B) of 7-day brainstem cells grown on laminin alone or CSPGs + laminin. Data represent at least 10 cells examined in complete serum-containing media.

We have also been characterizing the brainstem cells grown in culture to determine the extent of the classic serotonergic brainstem neuronal phenotype in our cultures. To date we have determined that a population of cells extending neurites in culture are neuronal (HuC positive) with some initial analysis indicating that a sub-population of these neurons are TPH2 (tryptophan hydoxylase) positive. Results for the TPH2 staining are currently being verified.

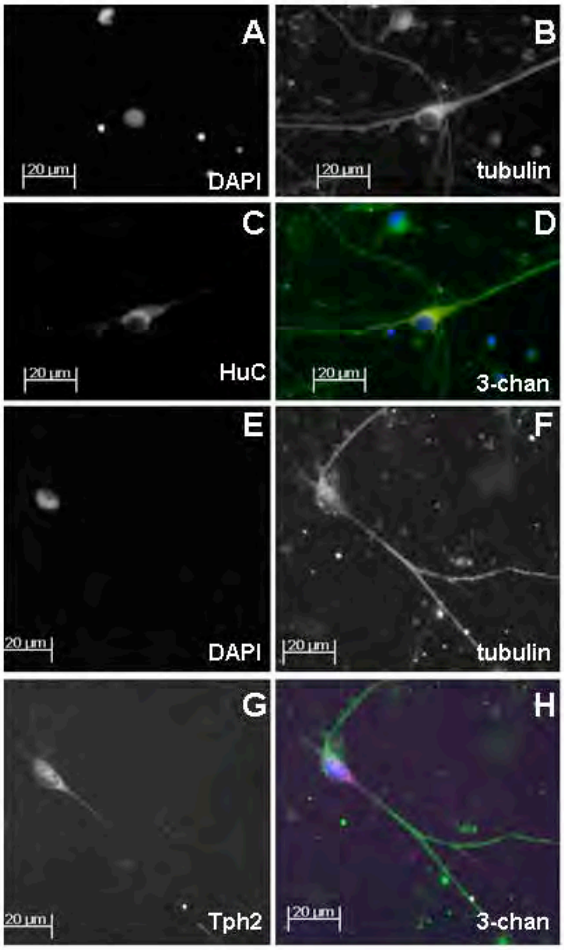


Figure 10. HuC and Tph2 characterization of cultured brainstem neurons.
(A-D) and (E-H) represent a single cell analyzed. DAPI staining of cell nucleus is shown in panels A and E. Tubulin neurite process labeling is shown in panels B and F. A HuC positive cell is shown in panel C. HuC is a neuronal specific marker evolutionarily conserved in many species including zebrafish. A note of added proof: sub-populations of cells in culture are also NeuN (neuronal marker) positive (data not shown). A Tph2 (tryptophan hydroxylase) positive cell is shown in panel G. Tph2 is the rate limiting enzyme in serotonin synthesis. Panels D and H are 3-channel merged images of A-C and E-G respectively.

SOW: Plunkett Laboratory

<u>Specific Aim 2:</u> To determine *in vitro* the involvement of L1.1 in axon growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates. Experiments will be carried out using established culture model systems to determine the extent of involvement of L1.1 in the ability of adult zebrafish brainstem neurons to grow their axon on substrates of chondroitin sulfates. L1.1 levels in cultured neurons will be perturbed using antisense morpholino and lentiviral vectors (to be generated in the Viral Vector Core facility at the University of Pittsburgh). There is ample evidence in the literature from in vivo and in vitro experiments that L1.1 (as well as its homolog in mammals, L1) is crucial for axon regeneration. Thus our data are physiologically relevant for human spinal cord injury/repair (Months 9-18).

Milestones:

Specific Aim 2.

Month 1-6: Prepare/optimize L1.1 antisense/control morpholino and LV-L1.1.
Month 7-12: Test morpholino and LV effects on L1.1 in neurons. Perform Experiments.

To determine the extent of involvement of L1.1 in the ability of adult zebrafish brainstem neurons to grow their axon on substrates of CS, we will perturb L1.1 levels in cultured neurons using antisense morpholino oligos. Two morpholinos were ordered from Gene Tools, LLC (www.gene-tools.com): (1) a fluoresceinated standard control 5'-CCTCTTACCTCAG TTACAATTTATA-3' and (2) a fluoresceinated morpholino directed against L1.1 5'-ATGAAA ACAGCCCCGACTCCAGACA-3', which was reported to significantly reduce L1.1 immunolabeling in vivo (Becker et al., 2004). To deliver morpholinos into cultured neurons, we are currently using Endo-porter (Gene Tools), a weak-base amphiphilic peptide that has been used to efficiently deliver morpholinos into cultured cells (Summerton, 2005). In preliminary experiments, we added the standard control morpholino to 96-hour adult brainstem neuronal cultures and have determined that control morpholino was delivered into some brainstem cells ***(Figure 11)****. Experiments are currently underway to optimize morpholino delivery. We will then test the effect of the L1.1 morpholino on neurons exposed to growth-permissive PDL/laminin (control) and growth-inhibitory CS.*

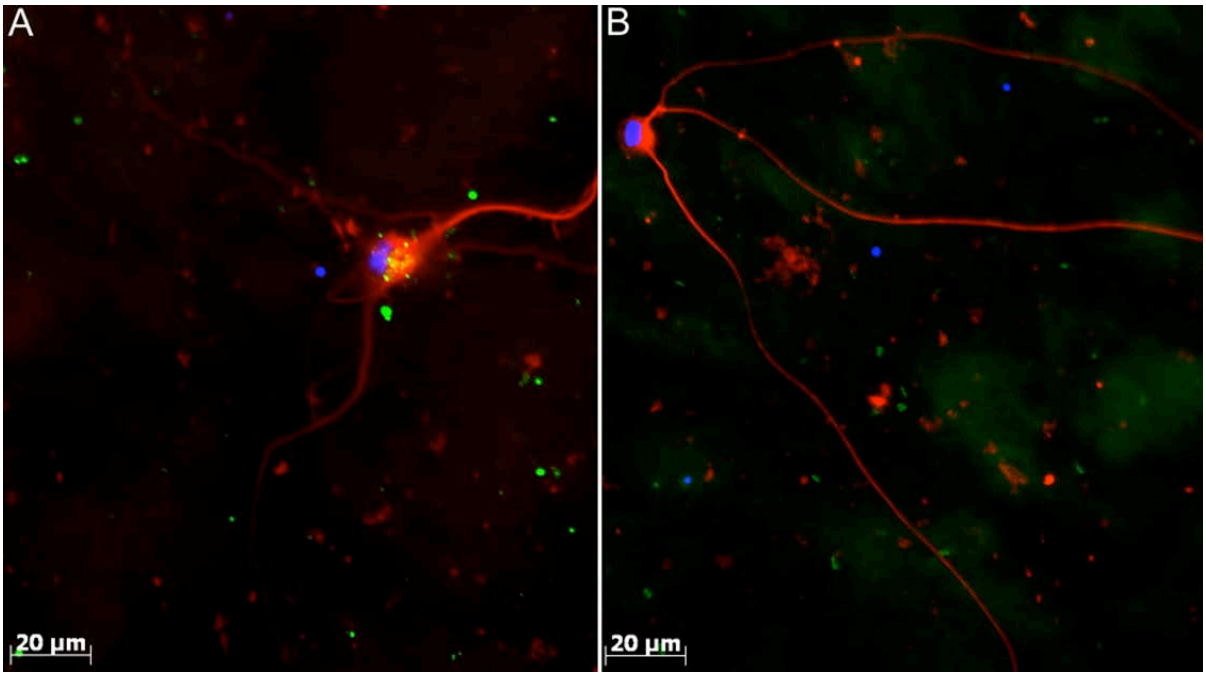


Figure 11. Delivery of control morpholinos into cultured brainstem cells
Neuronal processes are labeled with an anti-tubulin antibody and an Alexa-594 (red) secondary. Cell nuclei are labeled with DAPI (blue). Control morpholino labeled with fluorescein (green) is present in the cell body of the cell **(panel A)**. Panel B shows a cell from the same culture well that did not incorporate morpholino. Our results set the stage for delivery of L1.1 morpholinos.

Month 13-15: Analyze results Exp. #3. Start manuscript. Prepare follow-up experiment.

L1.1 Morpholino project
Experiments are currently underway to test the effect of the L1.1 morpholino on neurons exposed to growth-permissive PDL/laminin (control) and growth-inhibitory chondrotin sulfate proteoglycans. We have preliminary evidence that we have efficiently delivered L1.1 morpholino to our culture adult brainstem neurons. We have confirmed this data through the use of confocal microscopy which will give us an "inside" look as to delivery into the cell body. Below are fluorescent images of the delivery of FITC-tagged L1.1 morpholino.

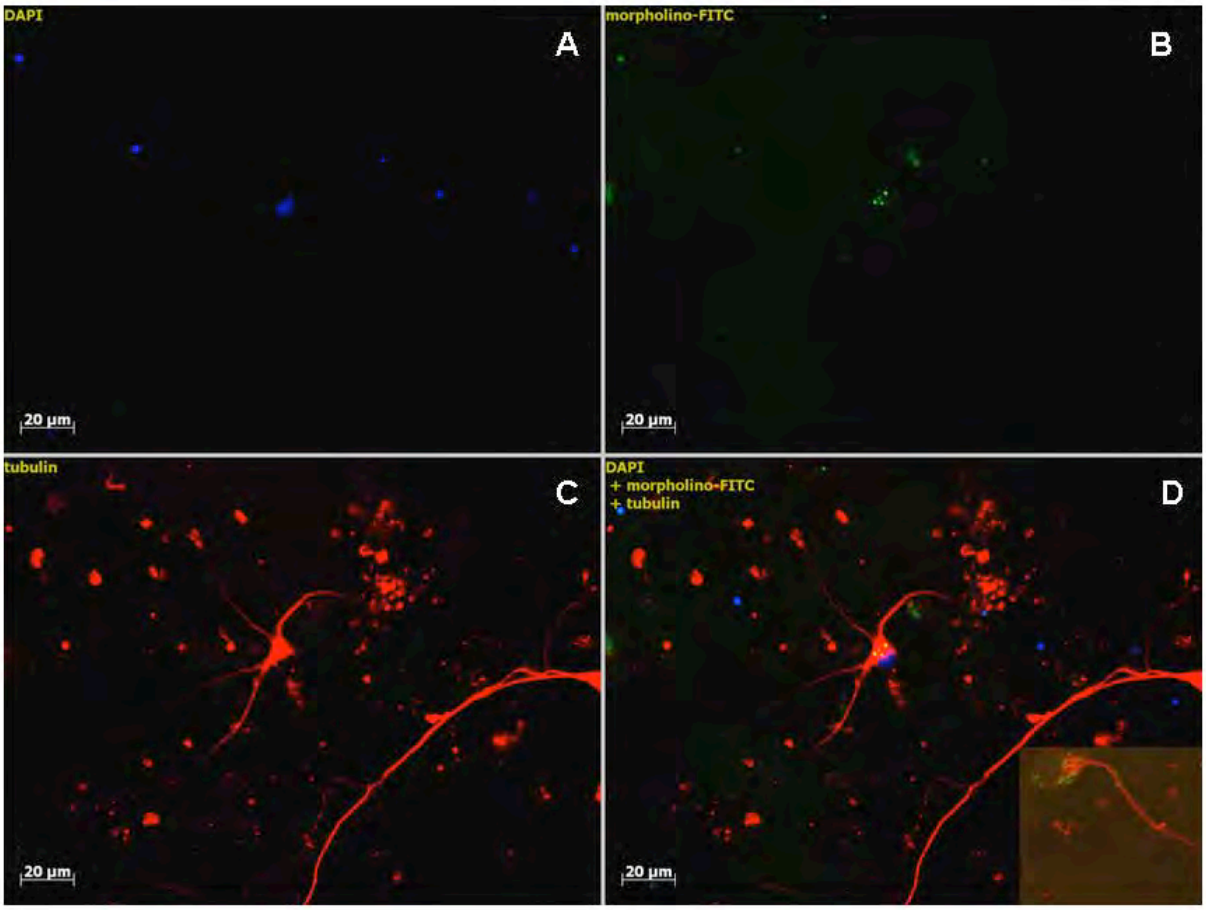


Figure 12. Delivery of L1.1 morpholinos into cultured brainstem cells
Cell nuclei are labeled with DAPI (blue) **(panel A).** L1.1 morpholino labeled with FITC is present in the cell body of the cell **(panel B).** Neuronal processes are labeled with an anti-tubulin antibody and an Alexa-594 (red) secondary **(panel C)**. Panel D shows an overlay image with the L1.1 FITC labeled morpholino co-localized with a neuronal cell body. Inset panel shows a confocal stack of 30/ 1uM images of L1.1 morpholino inside a brainstem neuron (arrowheads).

Having confirmed efficient delivery of L1.1 morpholino into cultured cells we will be able to begin to test our hypothesis and analyze the neurite outgrowth response of L1.1 knockdown cells in the presence of CSPGs.

L1.1 LV (upregulation) project

Full-length L1.1 cDNA-pGEM was obtained from Dr. Thomas Becker, University of Edinburgh (Wolman et. al., 2007). Bacteria were transformed and restriction digests were performed to verify the clone. The cDNA construct has been confirmed through sequencing. Full length L1.1 cDNA is currently being cloned into a Lenti-2A-EGFP vector (obtained from Dr. Vance Lemmon, University of Miami Medical School). This vector is useful for both lentiviral infection of cells and electroporation-mediated transfection of cells. In addition, the vector expresses enhanced GFP, which will allow us to distinguish between cultured neurons that carry the vector and those that do not. We will initially transfect primary brainstem cultures with L1.1-Lenti-2A-EGFP using electroporation-mediated transfection. Electroporation has been successfully used to express cDNAs in mammalian CNS neurons. This data will allow us to test our original hypothesis that neurons overexpressing L1.1 will exhibit better axon growth on CSPGs and that these axons have an increased ability to cross CSPG borders. Using electroporation, we currently are able to successfully transfect our brainstem neurons with a control pMax-GFP constructs and will use these parameters to transfect L1.1 constructs within the next month.

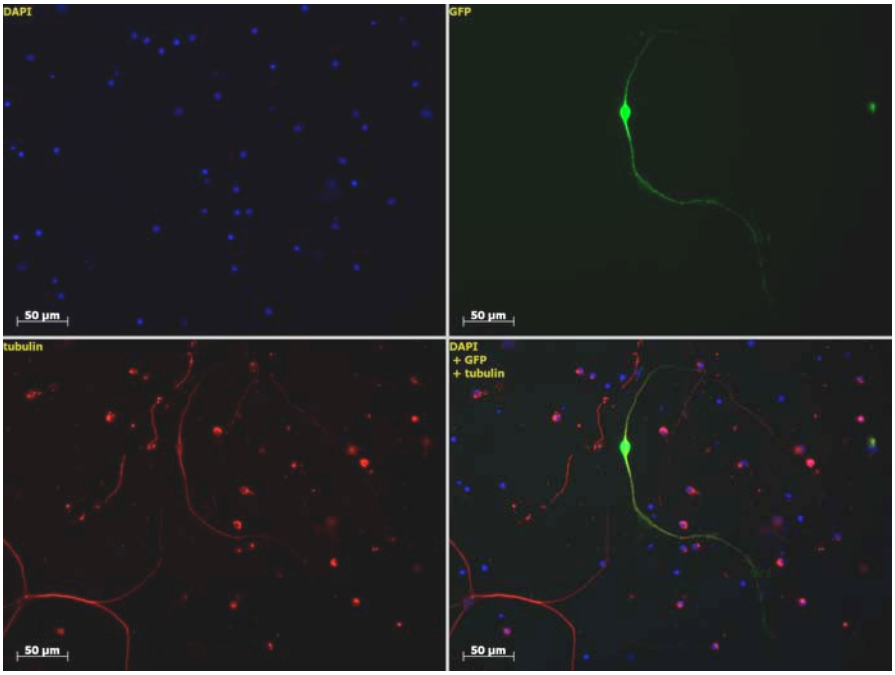


Figure 13. Five day zebrafish brainstem neurons transfected with 2 µg of pMax-GFP using Amaxa Basic Nucleofector Kit. (A-C) Individual images from three fluorescent channels. (A) Cell nuclei are labeled with DAPI (blue). (B) Transfected neuron expressing GFP. (C) Neuronal processes are labeled with an anti-tubulin antibody and Alexa-594 (red). (D) Composite derived from merging three channels.

SOW: Oudega Laboratory

Specifc Aim 3: To identify genes involved in axon regeneration from brainstem neurons in the injured adult zebrafish spinal cord.

Experiments will be performed to label brainstem neurons with fast blue that do or do not, regenerate an axon across an injury in the adult zebrafish spinal cord. Non-regenerators will be collected from the brainstem of zebrafish that received no spinal cord injury or an injury including a piece of Teflon to prevent any regeneration. Laser capture microdissection techniques will be used to collect these two neuron populations. Their mRNA will be isolated and used on zebrafish microarrays to reveal genes that are differentially expressed. We will reference our results to the gene database of the University of Kentucky (http://scigenes.uky.edu) and NCBI "Entrez Gene" (search axonal regeneration) www.ncbi.nlm.nih.gov database to allow us to determine genes important for axon regeneration. We will share out data with the NCBI Gene Expression Omnibus Database and the Allen spinal cord injury atlas if appropriate to allow fellow scientist to profit from the data. The known ability of zebrafish brainstem neurons to grow across CS-rich areas in the injured spinal cord makes them essentially different from rat neurons that are unable to do so. Even conditioned sensory neurons in rat are not able to grow across an established scar with CS present. Thus, the zebrafish spinal cord injury model allows us to study gene expression in neurons that regenerate their axon through an spinal cord injury milieu that has CS present as in mammals but through which mammalian neurons cannot extend their axon (Months 1-18).

Milestones:

Specific Aim 3. Dr.Katarina Vajn; Dr. Oudega's laboratory.

Month 1-6: Gain experience in spinal cord transection and tracing surgeries. Evaluate surgeries using histology. Practice LCM technique.

*In these first months a series of basic and necessary actions were completed and several supporting preliminary experiments were performed. The ACURO protocol for animal use was approved after some small changes and additionally a Postdoctoral Associate was identified, interviewed and subsequently hired. The Postdoctoral researcher is Katarina Vajn who received her MD and PhD degree from the University of Osijek in Croatia. Dr. Vajn has a background in spinal cord injury and will conduct the experiments described in Aim 3. We then started with testing several surgical approaches to transect the spinal cord in adult zebrafish in order to identify the most effective approach. The adult zebrafish spinal cord is approximately ~0.2 mm in diameter (Fig. 1**A**). It was important to establish the most optimal microsurgical techniques for spinal cord transection (Fig. 1**B**) and for neuronal tracer injections. The criteria for the tests that were conducted and compared* were 1) completeness of the transection, 2) minimal overall

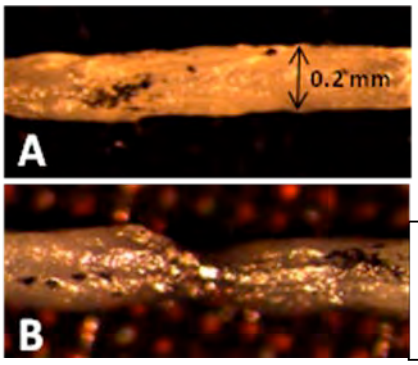


Fig. 1. Adult zebrafish spinal cord before (**A**) and after (**B**) complete transection. Spinal cord in panel B was harvested 1week after injury; some scar tissue has formed between the stumps. Spinal cords were fixed with PFA.

damage to the fish and the spinal cord and 3) long-term survival. We used histological stainings to analyze overall damage and assessed long-term survival. Based on the results we selected the least invasive surgical approach for injuring the spinal cord in the adult zebrafish.

Using our best surgical approach based on overall damage to the fish and the spinal cord, we observed the mortality rate in all tested approaches was relatively high and often variable. We found during execution of the first experiments that survival of zebrafish after a complete spinal cord transection was rather capricious and appeared to depend on a number of different variables such as water temperature and use of analgesics. We found out that a small deviation of the optimal temperature was enough to cause massive death among the operated fish. Our operated fish are housed in the Zebrafish facility of the University of Pittsburgh and are cared for by experienced caretakers. In addition, we found out that the use of analgesics after spinal cord transection as was recommended by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh was deleterious to the fish. We then on request of the IACUC tested different analgesics at different concentrations (Table 1). As can be seen none of the tested analgesics decreased the mortality rate below 100 %, whereas the omission of analgesics led to a mortality rate between 20-50 %. At completion of the tests we requested the IACUC to conduct our zebrafish experiments involving spinal cord transection without the use of analgesics. This request was approved by the IACUC and ACURO. Clearly, these first experiences with in vivo zebrafish spinal cord research will help us in the future but it delayed our experimentations drastically and cost literally hundreds of zebrafish and several months without making real scientific advances in our work.

Table 1. Overview of experiments in which different analgesics with different concentrations were used in spinal cord injured zebrafish. The fish received analgesic (column 2, 3) as listed in column 1 or not (column 4, 5). The mortality (percentage of fish that died within 48 h after surgery) is listed in gray-shaded columns 3 and 5.

zebrafish / ***analgesic***	*# with SCI + analgesic (experiment)*	*Mortality (percentage)*	*# with SCI - Analgesic (control)*	*Mortality (percentage)*
Butorphanol 0.5 mg/ml	*36*	*100*	*33*	*50*
Butorphanol 0.25 mg/ml	*42*	*100*	*37*	*24*
Carprofen 4 mg/ml	*25*	*100*	*29*	*42*
Carprofen 2 mg/ml	*32*	*100*	*32*	*29*
Carprofen 0.5 mg/ml	*46*	*100*	*43*	*20*

We then began to experiment with various retrograde neuronal tracers as each one is different and these tests were necessary to identify the most optimal tracer for our experiments. In addition, we started to investigate in detail the evolution of the injury-induced scar tissue. The results from that study would support execution of our time-course experiment for retrograde tracing.

Month 7-12: Start tracing brainstem neuronal subpopulations. Collect cells with LCM. Collect mRNA and prepare for microarrays. Hybridize microarrays (n=3).

*In this review period, we continued with the identification of the most optimal tracer and description of the evolution of the injury-induced scar tissue. The results from both studies were essential for optimal execution of the proposed labeling and harvesting of brainstem neurons that had and that hadn't regenerated their axon across a complete transection site in the spinal cord. Analysis of the injured zebrafish spinal cord at various times after transection was accomplished through the use histological techniques and immunocytochemistry (Fig. 2). We optimized several histological procedures such as perfusion/fixation, spinal cord/brain collection, and cryostat and paraffin sectioning. We then tested a variety of antibodies for their usefulness to stain zebrafish spinal cord. Examples of histological and immunocytochemical stainings are provided in figure 2. Horizontal cryostat sections were stained with cresyl violet to show the spinal cord cellular architecture (Fig. 2**A**). Others were stained with antibodies that recognize phosphocan, a axon growth-inhibitory chondroitin sulfate proteoglycan present in scar tissue as well as in normal tissue (Fig. 2**B**). We also developed double-staining techniques to demonstrate the relationship between NG2 (also a growth-inhibitory chondroitin sulfate proteoglycan) and glial-fibrillary acidic protein (GFAP) which is present in astrocytes (Fig. 2**C**). Precise information on the scar development will allow us to identify the best time to inject the tracers and correlate our labeling data with CS presence in the scar.*

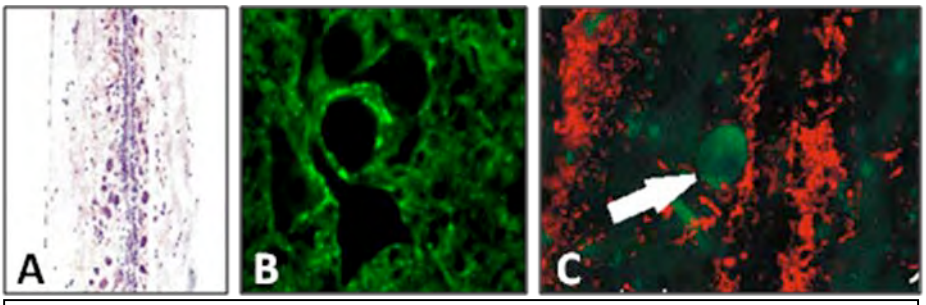


Fig 2. Three examples of staining preparations of adult zebrafish spinal cord tissue. The spinal cord was cut, harvested after 1 week and then fixed with paraformaldehyde, embedded, cut horizontally on a cryostat and then processed for staining. (**A**) Cresyl violet staining showing a concentration of cells including motor neurons at the spinal cord midline. (**B**) Phosphacan present in perineural nets. (**C**) NG2 (green) and GFAP (red) in double stained section. Arrow points to large motor neuron.

*We then began to investigate axon regeneration from brain stem neurons in time after a spinal cord transection. These data allowed us to determine the optimal time point for tracer injection. We studied the zebrafish brain atlas (Fig. 3**A**) in comparison to our brain sections (Fig. 3**B**) to start understanding the spatial relationships and dimensions of brainstem nuclei.*

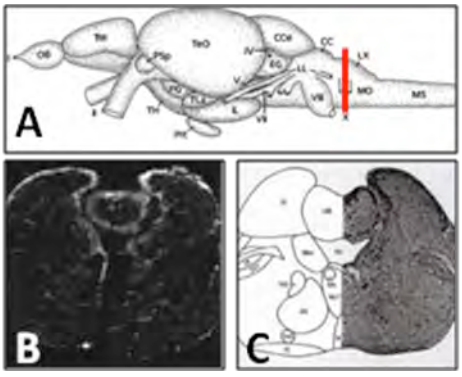


Fig. 3. Schematic drawing from the atlas of the adult zebrafish brain (**A**) in which the vertical red line indicates the level of the brainstem from which a transverse section is depicted in (**B**). Panel (**C**) provides the drawing from the zebrafish brain atlas with different nuclei indicated corresponding with the transverse section in (B).

*When collecting retrogradely labeled brainstem neurons we need to recognize the nuclei in our transverse brain tissue sections (Fig. 3**B**) compared to the atlas illustrations (Fig. 2**C**).*

We began our tracing studies with the well-known retrograde tracer Fast Blue, which is routinely used in our laboratory for tracing in rodent brain. We found this tracer less suitable for tracing in zebrafish brain with few neurons back-filled and relatively high non-specific fluorescent background staining (Fig. 4).

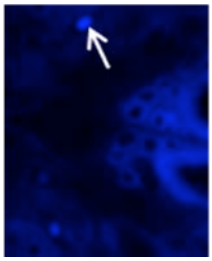


Fig 4. Transverse cryostat section of adult zebrafish brainstem after retrograde Fast Blue tracing from lower spinal cord levels. Arrow points at one of few back-filled neurons. Note abundant non-specific staining.

*We then tested a number of other tracers and found Fluoro-Emerald the most suitable (Fig. 5). One week after injection into the spinal cord Fluoro-Emerald is clearly visible in thicker (Fig. 5**A**) and thinner (Fig. 5**B**) axons at the caudal portion of the brainstem as well as in individual neurons (Fig. 5**C**).*

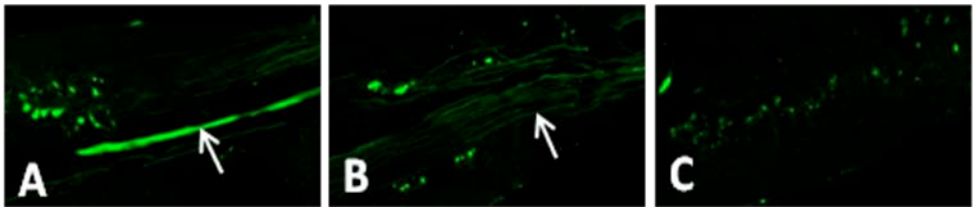


Fig. 5. Sagittal cryostat sections of adult zebrafish brainstem after retrograde tracing with Fluoro-Emerald from lower spinal cord levels. Sections were prepared 1 week after tracing. (**A**) Caudal brainstem and rostral spinal cord (to the left of the panel). Arrow points at a thick axon, likely derving from a Mautner cell. Also note the filled thinner axons. (**B**) Same areas as in A but different level. Arrow points at thinner axons filled with tracer. (**C**) Many tracer-filled cells are found in the intermediate reticular formation (NMLF).

Analysis of our Fluoro-Emerald spinal cord injections revealed nuclei of neurons back-filled with the green tracer (Fig 6A and 6B). We are currently assessing the total number of neurons back-filled with Fluoro-Emerald per zebrafish. This information will be essential for determining how many fish will need to be successfully retrogradely labeled to collect an adequate amount of brainstem neurons for microarray analysis.

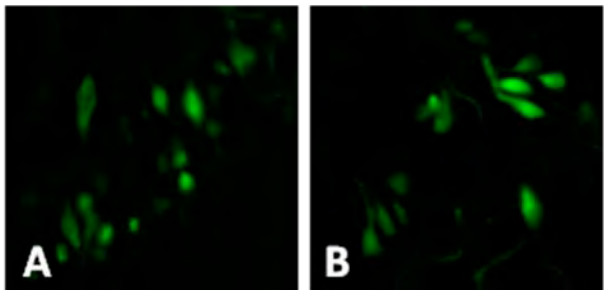


Fig. 6. Sagittal sections of zebrafish brainstem after Fluoro-Emerald tracing at 1 week after tracing. (**A**) Nucleus of neurons; some round and small other oval-shaped. (**B**) Nucleus with mostly oval-shaped neurons. Neurons in A are in the nucleus of the intermediate reticular formation (IMRF). Neurons in B are in the medial longitudinal fascicle (NMLF).

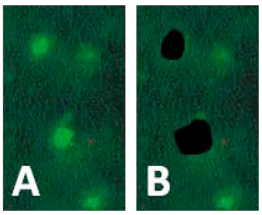


Fig. 7. Sagittal cryostat sections of adult zebrafish brainstem with green-labeled cells before (**A**) and after (**B**) LCM..

In parallel with our tracer experiments, we have established a collaboration with the laser capture microdissection (LCM) laboratory at the University of Pittsburgh. As shown in figure 7, we are currently able to extract cells using the LCM device. Backfilled neurons will be collected, mRNA isolated and subjected to analysis using microarrays.

We have started in this period to experiment with the basic and essential procedures to

execute these array experiments. To identify genes involved in axon regeneration from brainstem neurons in the injured adult zebrafish spinal cord, we will analyze the gene expression profile of two neuronal populations: (1) neurons that regenerate across an injury site and (2) neurons that have not undergone regeneration. Neurons will be collected from injured adult fish or control adult fish using laser capture microdissection techniques. Their mRNA will be isolated and then submitted for microarray analysis to identify genes that are differentially expressed. In preparation for these experiments, we have begun optimizing our RNA purification techniques. We have isolated RNA from adult zebrafish brains using Qiagen's RNeasy Plus Mini Kit. We have also performed reverse-transcriptase PCR reactions to assess the quality of our RNA using β-actin primers and the Superscript III First-Strand Synthesis system (Invitrogen). Establishment of these procedures is critical to the development of techniques used in microarray analysis.

Month 13-18: Analyze results. Start manuscript. Prepare follow-up experiment.

In this period of review, we have continued experiments to describe the development of the spinal cord injury site/scar following complete transection, as well as experiments to identify optimal conditions for neuronal tracing. We employed all surgical, histological, and immunocytochemical techniques developed in previous review periods. Zebrafish were injured, maintained for different periods of time, and then stained with cresyl violet to visualize cellular cytoarchitecture or with a battery of antibodies recognizing zebrafish homologs of proteoglycans, which are expressed in the mammalian glial scar. We observed proliferation of ependymoradial glial cells adjacent to the lesion site ***(Fig 8)****, and we detected expression of GFAP, an astrocyte marker, at the lesion site up to 8 weeks after surgery (when the experiment was terminated). CSPG's, in particular aggrecan and N2, were also expressed after spinal cord injury up to 8 weeks after surgery* ***(Figures 9-11)****. CSPGs* ***(Fig 12)*** *and aggrecan, in particular* ***(Fig 13)****, were also expressed in perineuronal nets rostral and caudal to the lesion site. NG2 is expressed in motorneurons before and after injury* ***(Fig 14).*** *These data were recently presented at the annual meeting of the Society for Neuroscience held in San Diego (November 2010). A poster entitled, "Molecular and cellular development of scar tissue in the injured spinal cord of adult zebrafish (Danio rerio)." K. Vajn, A. Tapanes-Castillo, F. Shabazz, JA Plunkett, and M. Oudega was presented by Dr. Vajn.*

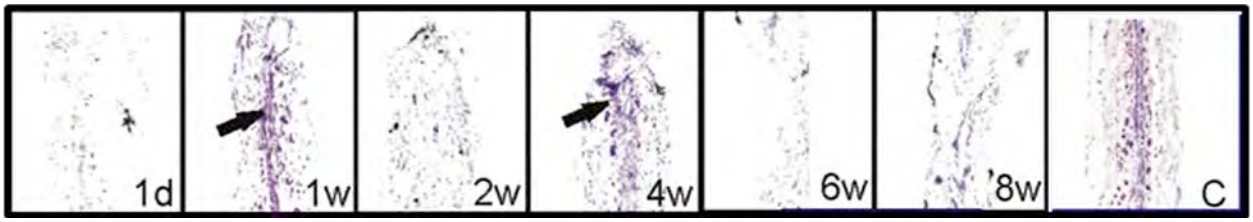


Fig. 8. Horizontal cresyl violet-stained sections. Ependymoradial glial cell proliferation adjacent to a spinal cord transection site increases over time. Ependymoradial glial cells (arrows) line the central canal.

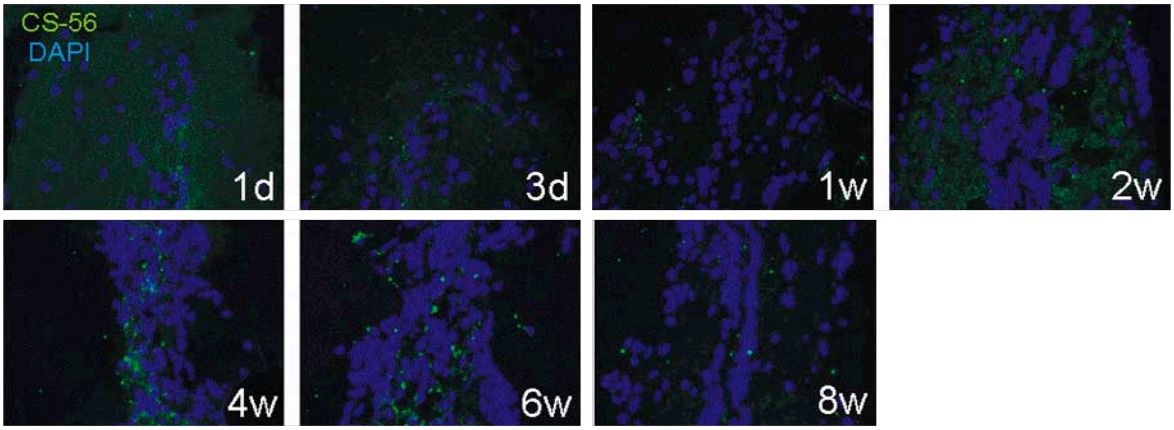


Fig. 9. CS-56 (green), an antibody which recognizes all CSPGs, is evident after injury in sections obtained from fish injured 1 day, 3 day, and 1-8 weeks prior to the termination of our experiment. In these panels, cell nuclei are stained blue with DAPI.

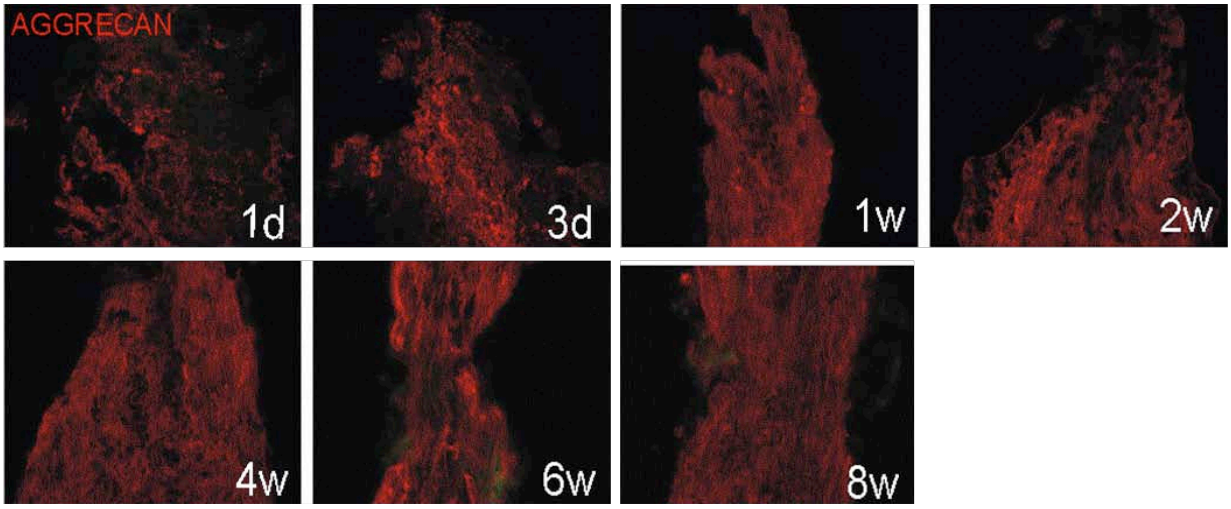


Fig. 10. Aggrecan, a CSPG family member, is expressed after injury in sections obtained from fish injured 1 day, 3 day, and 1-8 weeks prior to the termination of our experiment.

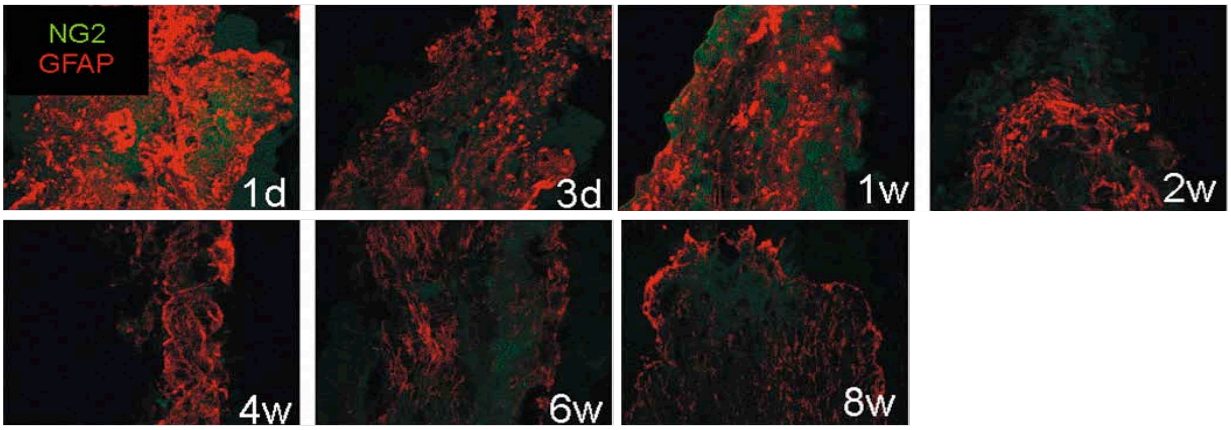


Fig. 11. GFAP, an astrocyte marker, and NG2, a CSPG family member, are expressed at the lesion site in sections obtained from fish injured 1 day, 3 day, and 1-8 weeks prior to the termination of our experiments.

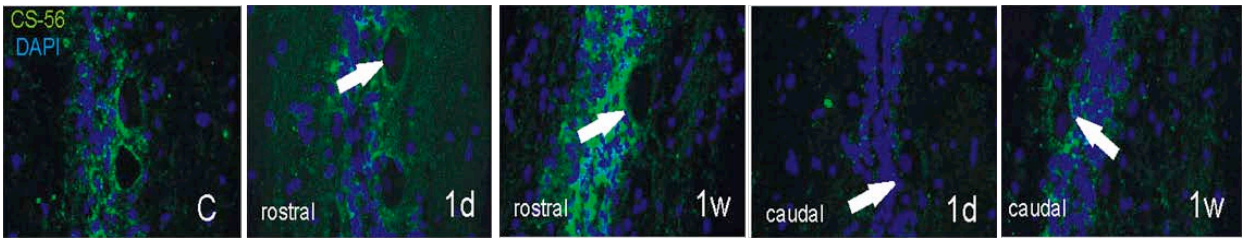


Fig. 12. CS-56 is expressed in perineuronal nets rostral and caudal to the lesion site.

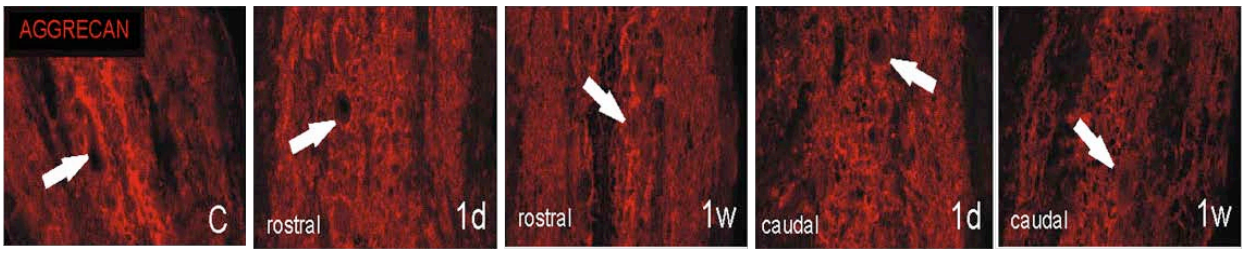


Fig. 13. Aggrecan is expressed in perineuronal nets rostral and caudal to the lesion site.

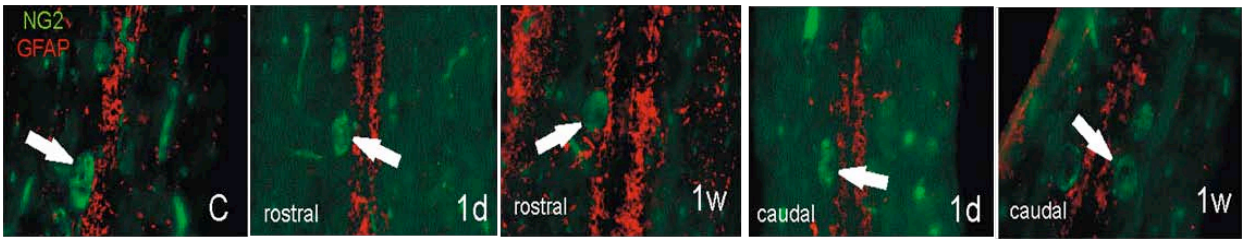


Fig. 14. NG2 is expressed in motorneurons throughout the spinal cord before and after spinal cord injury. This is an interesting observation. For the first time NG2 is found in motor neurons in the zebrafish. The role of NG2 in the normal functioning of motor neurons remains to be clarified.

Key Research Accomplishments:

Specific Aim 1 (Plunkett Lab)
(1) Established optimal brainstem culture and immunostaining conditions.

(2) Observed that cultured adult brainstem neurons respond differently to CSPGs.

(3) Cellular morphology characterization of culture system up to 14 days in vitro, including growth in serum-free media.

(4) Began to characterize cells in culture.

(5) Began quantifying neuronal response to CSPG data.

Specific Aim 2 (Plunkett Lab)
(1) Achieved control and L1.1 morpholino delivery into cultured cells.

(2) Currently delivering L1.1 morpholino into cells plated on CSPG to analyze changes in neurite outgrowth properties.

(2) Achieved electroporation delivery of control constructs into cultured cells.

(3) Currently cloning L1.1/Lenti vector.

Specific Aim 3 (Oudega Lab)

1. Established minimally invasive surgical techniques for spinal cord transection.

2. Established and optimized all techniques to acquire best immunocytochemical and histological staining.

3. Tested antibodies necessary to study spinal cord injury and its consequences.

4. Investigated the evolution of the glial scar after spinal cord transection. A manuscript is being prepared for publication in a peer-reviewd journal.

5. Started to investigate the time-course of axon regeneration beyond a transection in the spinal cord.

6. Established techniques to retrogradely label brainstem neurons.

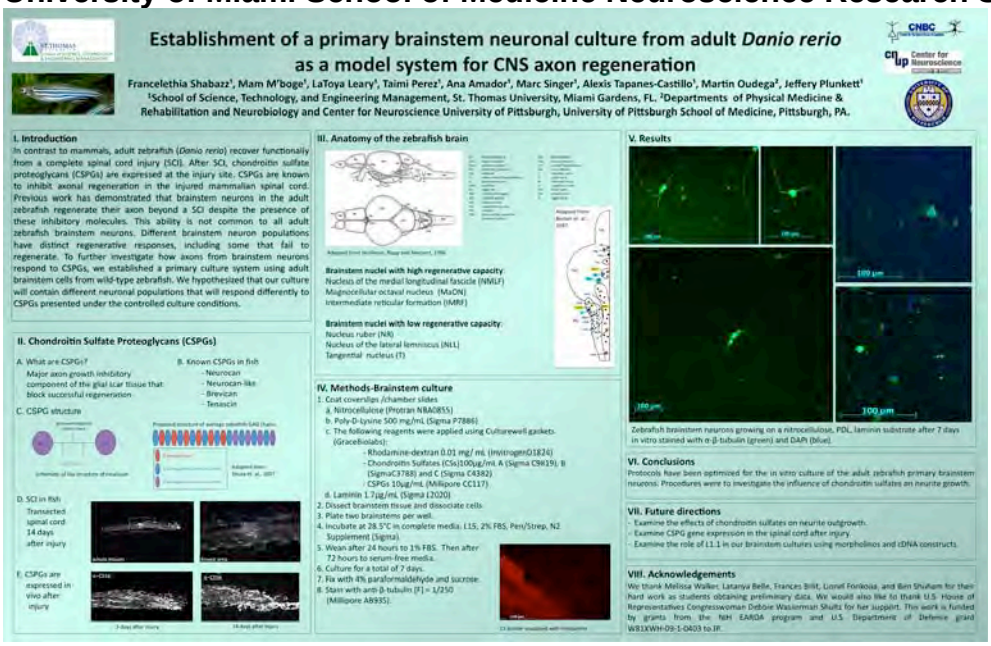
7. Established LCM techniques to harvest retrogradelylabeld neurions in adult zebrafish brainstem.

8. Tested crucial techniques such as reverse transcriptase PCR for gene expression studies in zebrafish CNS.

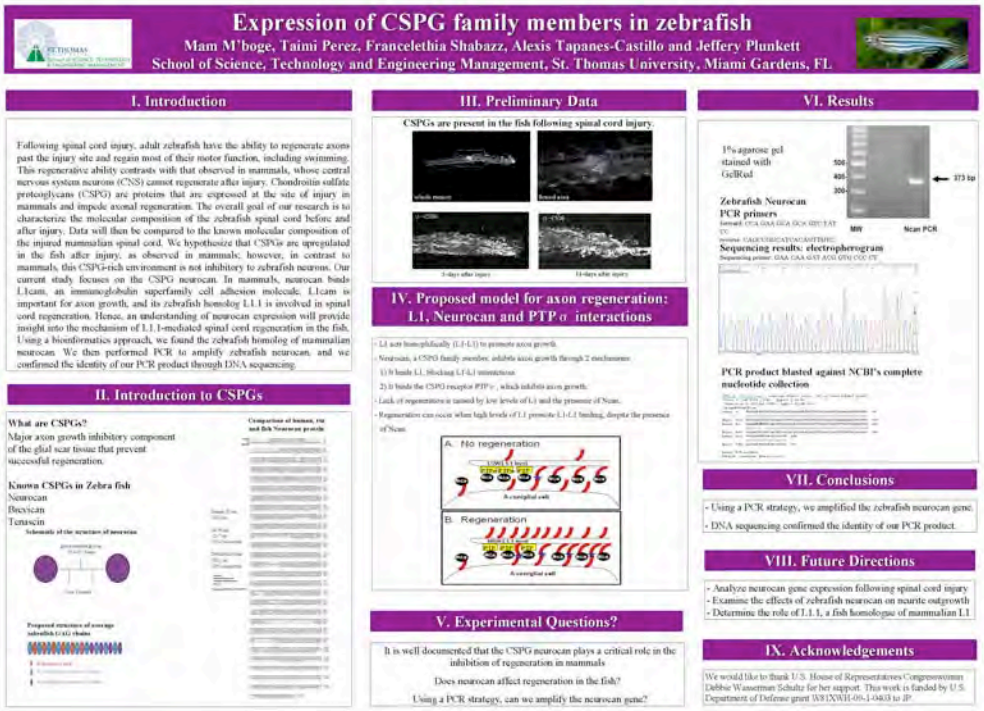
Reportable Outcomes:

Meeting Abstracts:
See Appendix below

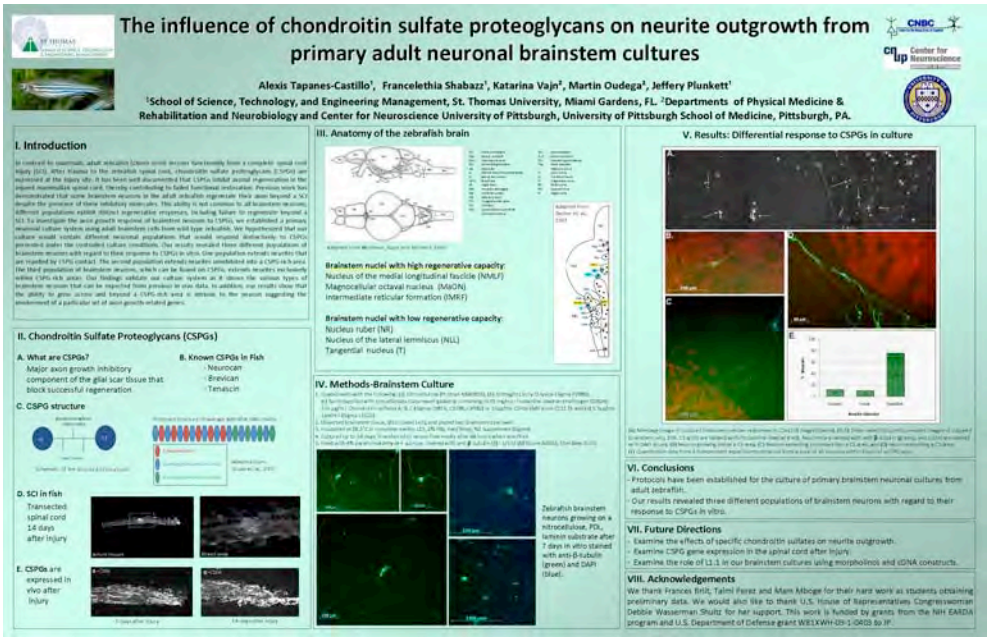
Posters presented:
University of Miami School of Medicine Neuroscience Research Symposium (Nov 2009)



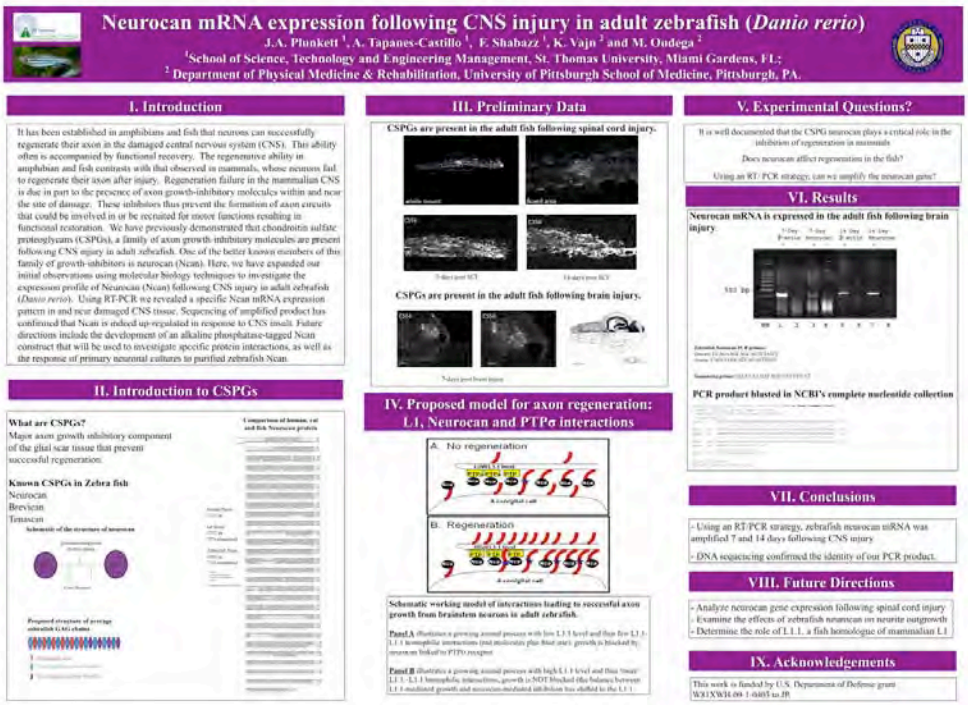
Southeast Florida Cell Science Undergraduate Research Symposium (April 2010)



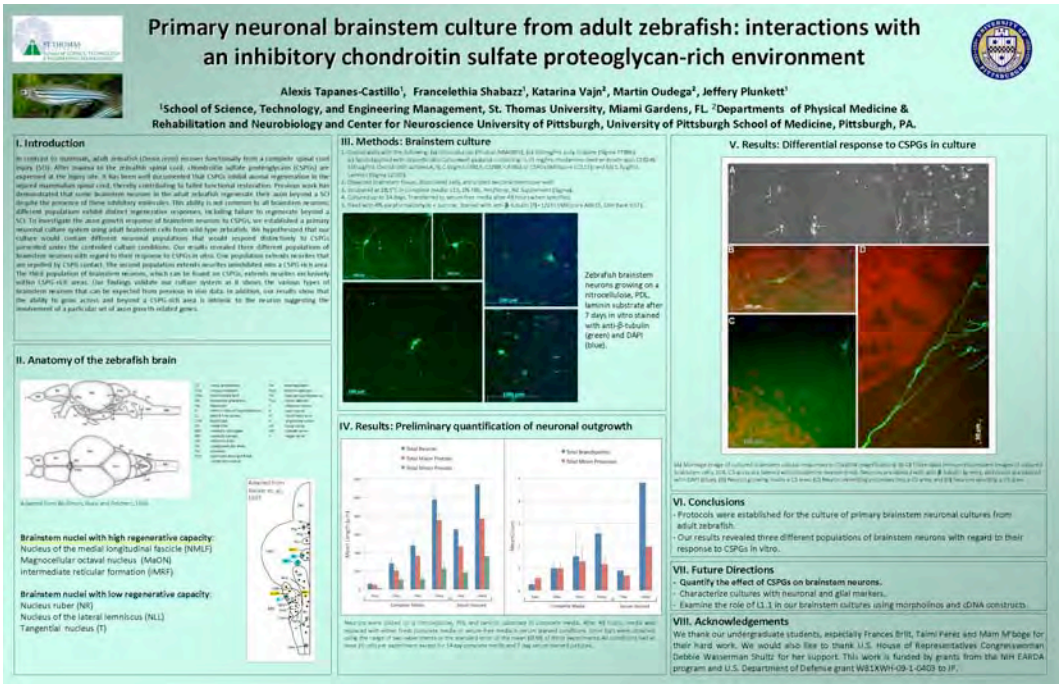
9th International Meeting on Zebrafish Development and Genetics (June 2010)



Society for Neuroscience, San Diego, Calif (Nov. 2010)



Society for Neuroscience, San Diego, Calif (Nov. 2010)



Primary neuronal brainstem culture from adult zebrafish: interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment

Alexis Tapanes-Castillo[1], Francelethia Shabazz[1], Katarina Vajn[2], Martin Oudega[2], Jeffery Plunkett[1]
[1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL. [2]Departments of Physical Medicine & Rehabilitation and Neurobiology and Center for Neuroscience University of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

II. Anatomy of the zebrafish brain

Brainstem nuclei with high regenerative capacity:
Nucleus of the medial longitudinal fascicle (NMLF)
Magnocellular octaval nucleus (MaON)
intermediate reticular formation (IMRF)

Brainstem nuclei with low regenerative capacity:
Nucleus ruber (NR)
Nucleus of the lateral lemniscus (NLL)
Tangential nucleus (T)

III. Methods: Brainstem culture

Zebrafish brainstem neurons growing on a nitrocellulose, PDL, laminin substrate after 7 days in vitro stained with anti-β-tubulin (green) and DAPI (blue).

IV. Results: Preliminary quantification of neuronal outgrowth

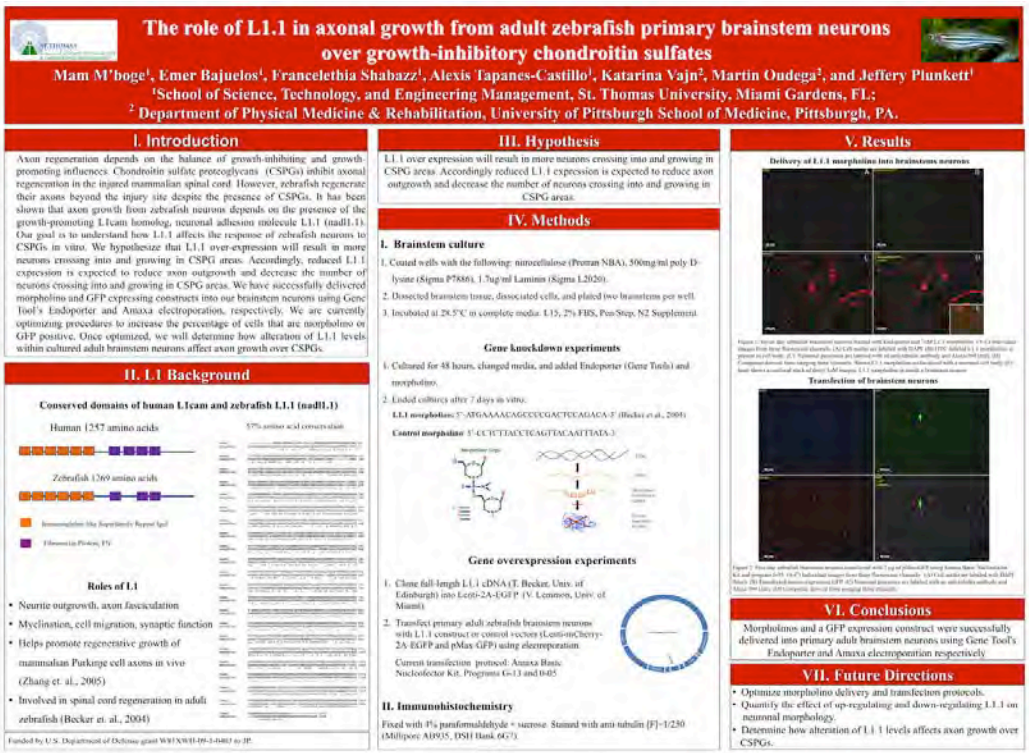
V. Results: Differential response to CSPGs in culture

VI. Conclusions
- Protocols were established for the culture of primary brainstem neuronal cultures from adult zebrafish.
- Our results revealed three different populations of brainstem neurons with regard to their response to CSPGs in vitro.

VII. Future Directions
- Quantify the effect of CSPGs on brainstem neurons.
- Characterize cultures with neuronal and glial markers.
- Examine the role of L1.1 in our brainstem cultures using morpholinos and cDNA constructs.

VIII. Acknowledgements
We thank our undergraduate students, especially Frances Britt, Talmi Perez and Mam M'boge for their hard work. We would also like to thank U.S. House of Representatives Congresswoman Debbie Wasserman Shultz for her support. This work is funded by grants from the NIH FARDA program and U.S. Department of Defense grant W81XWH-09-1-0403 to JP.

19th Neuroscience Symposium, University of Miami School of Medicine (Dec 2010)



The role of L1.1 in axonal growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates

Mam M'boge[1], Emer Bajuelos[1], Francelethia Shabazz[1], Alexis Tapanes-Castillo[1], Katarina Vajn[2], Martin Oudega[2], and Jeffery Plunkett[1]
[1]School of Science, Technology, and Engineering Management, St. Thomas University, Miami Gardens, FL;
[2] Department of Physical Medicine & Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh, PA.

I. Introduction

II. L1 Background

Conserved domains of human L1cam and zebrafish L1.1 (nadl1.1)

Human 1257 amino acids

Zebrafish 1269 amino acids

Roles of L1
- Neurite outgrowth, axon fasciculation
- Myelination, cell migration, synaptic function
- Helps promote regenerative growth of mammalian Purkinje cell axons in vivo (Zhang et. al., 2005)
- Involved in spinal cord regeneration in adult zebrafish (Becker et. al., 2004)

III. Hypothesis

L1.1 over expression will result in more neurons crossing into and growing in CSPG areas. Accordingly reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number of neurons crossing into and growing in CSPG areas.

IV. Methods

I. Brainstem culture
1. Coated wells with the following: nitrocellulose (Protran NBA), 500mg/ml poly D-lysine (Sigma P7886), 1.7ug/ml Laminin (Sigma L2020).
2. Dissected brainstem tissue, dissociated cells, and plated two brainstems per well.
3. Incubated at 29.5°C in complete media: L15, 2% FHS, Pen/Strep, N2 Supplement.

Gene knockdown experiments
1. Cultured for 48 hours, changed media, and added Endoporter (Gene Tools) and morpholino.
2. Ended cultures after 7 days in vitro.

L1.1 morpholino: 5'-ATGAAAACAGCCCGACTCCAGACA-3' (Becker et al., 2004)

Control morpholino: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

Gene overexpression experiments
1. Clone full-length L1.1 cDNA (T. Becker, Univ. of Edinburgh) into Lentí-2A-EGFP (V. Lemmon, Univ. of Miami).
2. Transfect primary adult zebrafish brainstem neurons with L1.1 construct or control vectors (Lentí-mCherry-2A-EGFP and pMax GFP) using electroporation.

Current transfection protocol: Amaxa Basic Nucleofector Kit. Programs G-13 and I-005

II. Immunohistochemistry

Fixed with 4% paraformaldehyde + sucrose. Stained with anti-tubulin (T)-1/250 (Millipore AB935, DSH Bank 6G7).

V. Results

Delivery of L1.1 morpholino into brainstem neurons

Transfection of brainstem neurons

VI. Conclusions

Morpholinos and a GFP expression construct were successfully delivered into primary adult brainstem neurons using Gene Tool's Endoporter and Amaxa electroporation respectively

VII. Future Directions
- Optimize morpholino delivery and transfection protocols.
- Quantify the effect of up-regulating and down-regulating L1.1 on neuronal morphology.
- Determine how alteration of L1.1 levels affects axon growth over CSPGs.

Funded by U.S. Department of Defense grant W81XWH-09-1-0403 to JP.

Student Success:
- Undergraduate students from St. Thomas University and the Plunkett Lab have or will be presenting their research at the following meetings:

- University of Miami School of Medicine Neuroscience Symposium (Nov 2009)
- 2nd Annual STEM Undergraduate Conference at Barry University (March 2010)
- Southeast Florida Cell Science Undergraduate Research Symposium (April 2010)
- 9th International Meeting on Zebrafish Development and Genetics (June 2010)
- Society for Neuroscience Meeting, San Diego, CA (Nov. 2010)
- University of Miami School of Medicine Neuroscience Symposium (Dec 2010)

This experience has enabled students to gain presentation experience. Undergraduate students have presented posters at 4 meetings in the last 12 months.

- Lionel Fonkoua a May 2009 graduate has been accepted to medical school at Penn State University and as of fall 2010 successfully completed his first semester.

- Frances Brlit a May 2009 graduate has been accepted to the DO program at Nova Southeastern University.

- Taimi Perez a May 2010 graduate has been accepted to the Summer Minority Student MCAT program at the University of Miami Miller School of Medicine.

- Francelethia Shabazz student and technician in the Plunkett Lab presented a talk entitled "The influence of chondroitin sulfate proteoglycans on neurite outgrowth from primary adult neuronal brainstem cultures" at the 5th Annual Southeast Florida Cell Science Undergraduate Research Symposium.

- The `annual Neuroscience Consortium meeting were held January 22, 2010 and October 1' 2010 at St. Thomas University. These meetings brought in consortium members from University of Pittsburgh to discuss progress of the project. Students and post-docs from both institutes benefited from the discussions concerning their particular aspect of the project.

- A manuscript entitled: Establishment and characterization of primary neuronal cultures from adult zebrafish brainstem. Tapanes-Castillo A, Vajn K, Shabazz F, Oudega M and Plunkett JA is in preparation to be submitted by spring 2011.

Conclusion:

The different studies within this proposal (in vitro as well as in vivo) have been progressing reasonably well according to the described milestones. Some technical/experimental barriers concerning specific aim 3 were encountered and these needed to be overcome. This was accomplished for most of them and is still in progress for few. Considering our previous success with surmounting these roadblocks, we are confident that we will be successful. Thus, in conclusion, we are well on our way to accomplish the goals as they were described in our proposal. With the continuation of this project in Phase 2 well underway we feel that our line of research will lead to many discoveries concerning the interaction of zebrafish neurites with chondroitin sulfates.

References:

Becker CG, Becker T (2002) Repellent guidance of regeneration optic axons by chondroitin sulfate glycosaminoglycans in zebrafish. J Neurosci 22(3): 842-853

Plunkett JA, Zambrano A, Fernandez L, Oudega M. (2006) Analysis of chondroitin sulfate proteoglycan expression in the transected zebrafish *Danio rerio* spinal cord. Soc Neurosci 31.

Appendices:

Poster Abstracts

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2009) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
\18th Annual University of Miami Miller School of Medicine Neuroscience Research Day, poster, University of Miami Miller School of Medicine, Miami, FL. (2009)

In contrast to mammals, adult zebrafish (*Danio rerio*) can recover from a central nervous system (CNS) injury and resume near normal swimming behavior about three months after a complete spinal cord transection. Strikingly, we found that recovery takes place despite the expression of chondroitin sulfate proteoglycans (CSPGs) at the injury site. CSPGs are known to be expressed in the CNS after injury and prevent axonal regeneration across the glial scar in mammals. However, since zebrafish brainstem neurons have the ability to regenerate axons past the site of injury, our data suggest that unlike mammals, zebrafish can regenerate CNS axons across a CSPG-rich environment. Yet, this ability is not common to all zebrafish brainstem neurons. It has been previously reported that different brainstem neuron populations have distinct regenerative responses: some regenerate after injury, while others do not. To further investigate how brainstem axons respond to CSPGs, we established a primary culture system using adult brainstem cells from wild-type zebrafish. We hypothesize that our culture will consist

of different neuronal populations that will respond differently to CSPGs presented under the controlled culture conditions. We expect some neurons will extend axons across CSPG-rich areas, while other neuronal populations will have their axons repelled by CSPGs. Future experiments aim to understand the genetic differences between brainstem neuron populations that exhibit distinct regenerative responses.

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2010) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
2nd Annual STEM Research Symposium, poster, Barry University, Miami Shores, FL. (2010)

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury (SCI). After SCI, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. CSPGs are known to inhibit axonal regeneration in the injured mammalian spinal cord. Previous work has demonstrated that brainstem neurons in the adult zebrafish regenerate their axon beyond a SCI despite the presence of these inhibitory molecules. This ability is not common to all adult zebrafish brainstem neurons. Different brainstem neuron populations have distinct regenerative responses, including some that fail to regenerate. To further investigate how axons from brainstem neurons respond to CSPGs, we established a primary culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture will contain different neuronal populations that will respond differently to CSPGs presented under the controlled culture conditions. *Supported by the NIH EARDA program and U.S. Department of Defense grant W81XWH-09-1-0403.*

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2010) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
5th Annual Southeast Florida Cell Science Undergraduate Research Symposium, poster, St. Thomas University, Miami Gardens, FL. (2010)

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury (SCI). After SCI, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. CSPGs are known to inhibit axonal regeneration in the injured mammalian spinal cord. Previous work has demonstrated that brainstem neurons in the adult zebrafish regenerate their axon beyond a SCI despite the presence of these inhibitory molecules. This ability is not common to all adult zebrafish brainstem neurons. Different brainstem neuron populations have distinct regenerative responses, including some that fail to regenerate. To further investigate how axons from brainstem neurons respond to CSPGs, we established a primary culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture will contain different neuronal populations that will respond differently to CSPGs presented under the controlled culture conditions. *Supported by the NIH EARDA program and U.S. Department of Defense grant W81XWH-09-1-0403.*

M’boge, M., Perez, T., Shabazz, F., Tapanes-Castillo, A. and Plunkett, J.A. (2010) Expression of CSPG family members in zebrafish.
5th Annual Southeast Florida Cell Science Undergraduate Research Symposium, poster, St. Thomas University, Miami Gardens, FL. (2010)

Following spinal cord injury, adult zebrafish have the ability to regenerate axons past the injury site and regain most of their motor function, including swimming. This regenerative ability contrasts with that observed in mammals, whose central nervous system neurons (CNS) cannot regenerate after injury. Chondroitin sulfate proteoglycans (CSPG) are proteins that are expressed at the site of injury in

mammals and impede axonal regeneration. The overall goal of our research is to characterize the molecular composition of the zebrafish spinal cord before and after injury. Data will then be compared to the known molecular composition of the injured mammalian spinal cord. We hypothesize that CSPGs are upregulated in the fish after injury, as observed in mammals; however, in contrast to mammals, this CSPG-rich environment is not inhibitory to zebrafish neurons. Our current study focuses on the CSPG neurocan. In mammals, neurocan binds L1cam, an immunoglobulin superfamily cell adhesion molecule. L1cam is important for axon growth, and its zebrafish homolog L1.1 is involved in spinal cord regeneration. Hence, an understanding of neurocan expression will provide insight into the mechanism of L1.1-mediated spinal cord regeneration in the fish. Using a bioinformatics approach, we found the zebrafish homolog of mammalian neurocan. We then performed PCR to amplify zebrafish neurocan, and we confirmed the identity of our PCR product through DNA sequencing.

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2010) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
9th Annual Student and Faculty Undergraduate Research Symposium, poster, St. Thomas University, Miami Gardens, FL. (2010)

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury (SCI). After SCI, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. CSPGs are known to inhibit axonal regeneration in the injured mammalian spinal cord. Previous work has demonstrated that brainstem neurons in the adult zebrafish regenerate their axon beyond a SCI despite the presence of these inhibitory molecules. This ability is not common to all adult zebrafish brainstem neurons. Different brainstem neuron populations have distinct regenerative responses, including some that fail to regenerate. To further investigate how axons from brainstem neurons respond to CSPGs, we established a primary culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture will contain different neuronal populations that will respond differently to CSPGs presented under the controlled culture conditions. *Supported by the NIH EARDA program and U.S. Department of Defense grant W81XWH-09-1-0403.*

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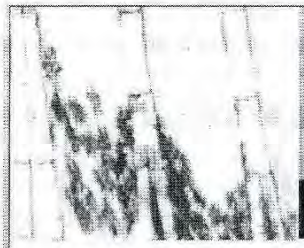
Following spinal cord injury, adult zebrafish have the ability to regenerate axons past the injury site and regain most of their motor function, including swimming. This regenerative ability contrasts with that observed in mammals, whose central nervous system neurons (CNS) cannot regenerate after injury. Chondroitin sulfate proteoglycans (CSPG) are proteins that are expressed at the site of injury in mammals and impede axonal regeneration. The overall goal of our research is to characterize the molecular composition of the zebrafish spinal cord before and after injury. Data will then be compared to the known molecular composition of the injured mammalian spinal cord. We hypothesize that CSPGs are upregulated in the fish after injury, as observed in mammals; however, in contrast to mammals, this CSPG-rich environment is not inhibitory to zebrafish neurons. Our current study focuses on the CSPG neurocan. In mammals, neurocan binds L1cam, an immunoglobulin superfamily cell adhesion molecule. L1cam is important for axon growth, and its zebrafish homolog L1.1 is involved in spinal cord regeneration. Hence, an understanding of neurocan expression will provide insight into the mechanism of L1.1-mediated spinal cord regeneration in the fish. Using a bioinformatics approach, we found the zebrafish homolog of mammalian neurocan. We then performed PCR to amplify zebrafish neurocan, and we confirmed the identity of our PCR product through DNA sequencing.



9th International Meeting on
ZEBRAFISH
DEVELOPMENT AND GENETICS
WEDNESDAY, JUNE 16 - SUNDAY, JUNE 20, 2010

1. Enter Authors | 2. Enter Abstract Title and Topic | 3. Enter Abstract Body | **4. Review**

Abstract Review: Please print a copy of this page for your records

Below is an approximation of what your abstract will look like in the abstract book. Please print a copy of this page for your records.

The Influence of Chondroitin Sulfate Proteoglycans on Neurite Outgrowth from Primary Adult Neuronal Brainstem Cultures

Alexis Tapanes-Castillo[1], *Francelethia Shabazz*[1], *Katarina Vajn*[2], *Martin Oudega*[2], *Jeffery A. Plunkett*[1]

[1] St. Thomas University, Miami Gardens, FL, USA, [2] University of Pittsburgh, Pittsburgh, PA, USA

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury (SCI). After trauma to the zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. It has been well documented that CSPGs inhibit axonal regeneration in the injured mammalian spinal cord, thereby contributing to failed functional restoration. Previous work has demonstrated that some brainstem neurons in the adult zebrafish regenerate their axon beyond a SCI despite the presence of these inhibitory molecules. This ability is not common to all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond a SCI. To investigate the axon growth response of brainstem neurons to CSPGs, we established a primary neuronal culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs presented under the controlled culture conditions. Our results revealed three different populations of brainstem neurons with regard to their response to CSPGs in vitro. One population extends neurites that are repelled by CSPG contact. The second population extends neurites uninhibited into a CSPG-rich area. The third population of brainstem neurons, which can be found on CSPGs, extends neurites exclusively within CSPG-rich areas. Our findings validate our culture system as it shows the various types of brainstem neurons that can be expected from previous in vivo data. In addition, our results show that the ability to grow across and beyond a CSPG-rich area is intrinsic to the neuron suggesting the involvement of a particular set of axon growth-related genes.

☐ I have read and approve of my abstract as printed above

Finish

The following three abstracts were submitted for the November 2010 Society for Neuroscience meeting in San Diego, California.

Control/Tracking Number: 2010-S-6281-SfN
Primary neuronal brainstem culture from adult zebrafish: interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment

AUTHOR BLOCK: A. TAPANES-CASTILLO[1], F. SHABAZZ[1], K. VAJN[2], M. OUDEGA[2], *J. A. PLUNKETT[1];
[1]St Thomas Univ., Miami Gardens, FL; [2]Univ. of Pittsburgh, Pittsburgh, PA
Abstract:
In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury. After trauma to the zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. It has been well documented that CSPGs inhibit axonal regeneration in the injured mammalian spinal cord, which contributes to the lack of endogenous functional restoration. Previous work in our laboratory has demonstrated that brainstem neurons in the adult zebrafish can regenerate their axon beyond a spinal cord lesion despite the presence of these inhibitory molecules. This ability is not characteristic for all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site. To investigate the axonal growth response of zebrafish brainstem neurons to CSPGs, we developed a primary neuronal culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs presented under controlled culture conditions. Our results supported this hypothesis revealing three different populations of brainstem neurons with regard to their response to CSPGs in vitro. One population outside of CSPG-rich areas extends neurites that are repelled upon contact with CSPGs. Another population outside of CSPG-rich areas extends neurites that grow into and across the CSPG environment. The third population remains exclusively within CSPG-rich areas and extends neurites across CSPG-rich areas. Our results suggest that the ability to grow across and beyond a CSPG-rich area is intrinsic to the neuron. This ability or disability to grow across CSPGs likely involves unique sets of axon growth-related genes.
This work is supported by United States Department of Defense grant W81XWH-09-1-0403 to JAP.

Control/Tracking Number: 2010-S-6409-SfN
Activity: Scientific Abstract
Current Date/Time: 5/11/2010 12:04:02 PM

Neurocan mRNA expression following CNS injury in adult zebrafish (Danio rerio)

AUTHOR BLOCK: J. A. PLUNKETT[1], *A. TAPANES-CASTILLO[1], F. SHABAZZ[1], K. VAJN[2], M. OUDEGA[2];
[1]Sch. of Sci., St. Thomas Univ., MIAMI, FL; [2]Univ. of Pittsburgh, Pittsburgh, PA

Abstract: It has been established in amphibians and fish that neurons can successfully regenerate their axon in the damaged central nervous system (CNS). This ability often is accompanied by functional recovery. The regenerative ability in amphibian and fish contrasts with that observed in mammals, whose neurons fail to regenerate their axon after injury. Regeneration failure in the mammalian CNS is due in part to the presence of axon growth-inhibitory molecules within and near the site of damage. These inhibitors thus prevent the formation of axon circuits that could be involved in or be recruited for motor functions resulting in functional restoration. We have previously demonstrated that chondroitin sulfate proteoglycans (CSPGs), a family of axon growth-inhibitory molecules are present following CNS injury in adult zebrafish. One of the better known members of this family of growth-inhibitors is neurocan (Ncan). Here, we have expanded our initial observations using molecular biology techniques to investigate the expression profile of Neurocan (Ncan) following CNS injury in adult zebrafish (Danio rerio). Using RT-PCR we revealed a specific Ncan mRNA expression pattern in and near damaged CNS tissue. Sequencing of amplified product has confirmed that Ncan is indeed up-regulated in response to CNS insult. Future directions include the development of an alkaline phosphatase-tagged Ncan construct that will be used to investigate specific protein interactions, as well as the response of primary neuronal cultures to purified zebrafish Ncan.
This work is supported by United States Department of Defense grant W81XWH-09-1-0403 to JAP.
:

Presentation Preference (Complete): Poster Only
Linking Group (Complete): MeadowGold
Nanosymposium Information (Complete):
Theme and Topic (Complete): C.10.d. Spinal cord: Cellular and molecular mechanisms
Keyword (Complete): ZEBRAFISH ; PROTEOGLYCAN ; REGENERATION
Support (Complete):
Support: Yes
Grant/Other Support: : DOD Grant W81XWH-09-1-0403

Special Requests (Complete):
Is the first (presenting) author of this abstract a high school or undergraduate student?: No

Religious Conflict?: No Religious Conflict
Additional Conflict?: No

Status: Finalized

Vajn, K., Tapanes-Castillo, A., Shabazz, F., **Plunkett, J.A**., Oudega, M. (2010) Molecular and cellular development of scar tissue in the injured spinal cord of adult zebrafish (*Danio rerio*). 40th Annual Society for Neuroscience Meeting, San Diego, CA.

Adult zebrafish (*Danio rerio*) recover from functional impairments after spinal cord injury (SCI), which is due at least in part to successful regeneration of brainstem axons across and beyond the injury site. This remarkable restorative ability of zebrafish is in sharp contrasts with the failure to recover function seen after SCI in adult mammals. One of the mechanisms underlying this inability is the expression of growth-inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) in scar tissue at the site of injury, which, in concert with the cellular architecture of the scar, obstruct axonal growth and thus the formation of axonal circuits that could be involved in functional recovery. One possible explanation for successful repair after SCI in adult zebrafish may be that the scar does not develop into an obstructive barrier for regenerating axons. Currently, very little is known about the molecular and cellular development of scar tissue in the injured spinal cord in adult zebrafish. In the present study we analyzed the temporal expression profiles of several members of the family of CSPGs in the transected spinal cord of adult zebrafish. Among the studied CSPGs are NG2, versican, brevican, neurocan, and phosphocan. NG2 expression was decreased at the lesion site but remained highly expressed by motoneurons throughout the spinal cord. The expression profiles of the other CSPGs also revealed distinct characteristics. These results together with the developing scar cytoarchitecture may reveal key aspects explaining the observed anatomical and functional repair after SCI in adult zebrafish. This work is supported by United States Department of Defense grant W81XWH-09-1-0403 to JAP.

19th Neuroscience Symposium, University of Miami School of Medicine (Dec 2010)

The role of L1.1 in axon growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates.

Mam M'boge[1], Emer Bajuelos[1], Fran Shabazz[1], Alexis Tapanes-Castillo[1], Katarina Vajn[2], Martin Oudega[2] and Jeffery Plunkett[1]

[1]School of Science, Technology and Engineering Management, St. Thomas University, Miami, FL
[2] Departments of Physical Medicine and Rehabilitation and Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Axon regeneration depends on the balance of growth-inhibiting and growth-promoting influences. It has been shown that axon growth from zebrafish neurons depends on the presence of the growth-promoting L1 homolog, L1.1. Moreover, the extent of axon growth is directly correlated with the level of L1.1. Chondroitin sulfate proteoglycans (CSPGs) inhibit axonal regeneration in the injured mammalian spinal cord. However, zebrafish neurons regenerate their axons beyond the injury site despite the presence of CSPGs. Our goal is to understand how L1.1 affects the response of zebrafish brainstem neurons to CSPGs *in vitro*. We hypothesize that L1.1 overexpression will result in more neurons crossing into and growing in CSPG areas. Accordingly, reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number neurons crossing into and growing in CSPG areas. We have successfully delivered morpholinos and GFP expressing constructs into our brainstem neurons using Gene Tool's Endoporter and Amaxa electroporation respectively. We are currently optimizing procedures to increase the percentage of cells that are morpholino/GFP positive. Once optimized, we will determine how alteration of L1.1 levels within cultured adult brainstem neurons affects axon growth over CSPGs.

The following abstracts have been accepted to the 75th Annual Meeting of the Florida Academy of Sciences Meeting in Melbourne, Florida, March 11-12, 2011

Primary neuronal brainstem culture from adult zebrafish: interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment.

A. TAPANES-CASTILLO (1), F. SHABAZZ (1), K. VAJN (2), M. OUDEGA (2), and J. PLUNKETT (1). (1) School of Science, Technology, and Engineering Management, St. Thomas University, 16401 NW 37th Avenue, Miami Gardens, FL 33054; (2) Center for Neuroscience, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15213.

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury. After trauma to the zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. It has been well documented that CSPGs inhibit axonal regeneration in the injured mammalian spinal cord, which contributes to the lack of endogenous functional restoration. Previous work in our laboratory has demonstrated that brainstem neurons in the adult zebrafish can regenerate their axon beyond a spinal cord lesion despite the presence of these inhibitory molecules. This ability is not characteristic for all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site. To investigate the axonal growth response of zebrafish brainstem neurons to CSPGs, we developed a primary neuronal culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs presented under controlled culture conditions. Our results supported this hypothesis revealing three different populations of brainstem neurons with regard to their response to CSPGs in vitro. One population outside of CSPG-rich areas extends neurites that are repelled upon contact with CSPGs. Another population outside of CSPG-rich areas extends neurites that grow into and across the CSPG environment. The third population remains exclusively within CSPG-rich areas and extends neurites across CSPG-rich areas. Our results suggest that the ability to grow across and beyond a CSPG-rich area is intrinsic to the neuron. This ability or disability to grow across CSPGs likely involves unique sets of axon growth-related genes. (Funded by U.S. Dept. of Defense W81XWH-09-1-0403 to JP).

The role of L1.1 in axonal growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates.

M. M'BOGE (1), E. BAJUELOS (1), F. SHABAZZ (1), A. TAPANES-CASTILLO (1), K. VAJN (2), M. OUDEGA (2), and J. PLUNKETT (1). (1) School of Science, Technology, and Engineering Management, St. Thomas University, 16401 NW 37th Avenue, Miami Gardens, FL 33054; (2) Center for Neuroscience, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15213.

Axon regeneration depends on the balance of growth-inhibiting and growth- promoting influences. Chondroitin sulfate proteoglycans (CSPGs) inhibit axonal regeneration in the injured mammalian spinal cord. However, zebrafish regenerate their axons beyond the injury site despite the presence of CSPGs. It has been shown that axon growth from zebrafish neurons depends on the presence of the growth-promoting L1cam homolog, neuronal adhesion molecule L1.1 (nadl1.1). Our goal is to understand how L1.1 affects the response of zebrafish neurons to CSPGs in vitro. We hypothesize that L1.1 over-expression will result in more neurons crossing into and growing in CSPG areas. Accordingly, reduced L1.1 expression is expected to reduce axon outgrowth and decrease the number of neurons crossing into and growing in CSPG areas. We have successfully delivered morpholino and GFP expressing constructs into our brainstem neurons using Gene Tool's Endoporter and Amaxa electroporation, respectively. We are currently optimizing procedures to increase the percentage of cells that are morpholino or GFP positive. Once optimized, we will determine how alteration of L1.1 levels within cultured adult brainstem neurons affect axon growth over CSPGs. (Funded by U.S. Dept. of Defense W81XWH-09-1-0403 to JP).

ORAL PRESENTATION

Shabazz, F. and Plunkett JA (2010) The influence of chondroitin sulfate proteoglycans on neurite outgrowth from primary adult neuronal brainstem cultures.
5th Annual Southeast Florida Cell Science Undergraduate Research Symposium, oral presentation, St. Thomas University, Miami Gardens, FL.